

What greenhouse convention?

Norway has been playing host to the first of four UN regional conferences, and has been abuzz with greenhouse talk. But the problem of negotiating what to do about the greenhouse effect is as far from solved as ever.

BERGEN is not usually a busy place, but the past two weeks have been exceptional. One of four regional conferences at ministerial level in preparation for a UN conference in 1992 was preceded by a more general discussion among 150 scientists, with the blessing of such organizations as the Norwegian Research Council and the European Science Foundation. The proceedings appear to have been admirably measured — more so than, for example, the report two years ago of the commission on world environmental problems of which Mrs Gro Harlem Bruntland, lately prime minister of Norway, was the chairman. As usual on these occasions (see page 193), an environmental pressure group did its best to enliven the proceedings by publishing the text of a US embassy document.

The central question last week was the prospect of global warming as a consequence of an excess greenhouse effect. That is as it should be. The conference statement valuably breaks new ground by explaining to its readers why uncertainty (about the timing or magnitude of the effect) is not an excuse for doing nothing, and goes on to advocate the negotiation of a convention to regulate the emission of greenhouse gases. The statement is even enlightened enough to acknowledge that the nature of the problems that must be solved before a convention can stick is partly economic and partly a matter of social equity, national and international. Fine. More of that, and we shall all be the wiser.

So how to negotiate a convention? The essential trick is to persuade everybody that it is needed before anybody knows how it would cramp his style. Of necessity, the participants must be governments. The people sitting round the table will be their representatives. The best that could be done in the next few years (say three) would be an agreement to agree. An agreement to agree on terms made explicit will take longer. Unilateral declarations of economic self-deprivation in the meantime will earn no brownie points. Abuse of the United States for not being in the vanguard of self-abnegation will not merely serve no purpose, it will be counterproductive. (And has China yet been asked?) The Bergen meeting showed that there is plenty that academics can meanwhile do, but they must learn that it is politicians who must eventually sign the convention that they advocate. □

If the agenda is to save the surface of the Earth for posterity, or even merely to rub along until posterity is

smarter than its predecessors, intelligent compromise will be necessary at every step, chiefly between rich and poor. The politics of cataclysmic populism will get us nowhere. □

Animals at work

Accusations of animal abuse have shocked the British public. But the news should have come from within.

ALREADY demoralized, Britain's biology researchers will find little comfort in last week's allegations by an animal-welfare group that Professor Wilhelm Feldberg has broken the laws on animal experimentation (see page 190). Scientific research in Britain is already enough in the doldrums; the extra opprobrium that could now accrue will not much help good causes. It is especially damaging that the alleged violations should have happened at the Medical Research Council's largest in-house laboratory. The best hope must be that the inquiries promised by the council and the Home Office will reveal that this is an isolated incident.

On an issue so complex ethically, researchers cannot afford to be complacent. The topic is one that excites general concern among the public, for many of whom the unavoidable use of animals in research is their sole knowledge of scientific matters. Britain has already had an unwelcome taste of violence and threats thereof against researchers and their laboratories. The task of winning round opinion will be made more difficult if the impression (however unfounded) gains ground that the provisions of recent legislation can be ignored.

There is no doubt that most researchers take their ethical responsibilities to animals seriously. How can that be demonstrated? It is disturbing that there are individual researchers who dare not say openly that 20 Home Office inspectors for 18,000 licence holders are not enough effectively to police the legislation, or that the inspectors may not be properly qualified. But too much is at stake to rely on the assurance of government inspectors. Laboratories, in their own interests, should look out for themselves — some senior person should supervise all animal experimentation, should be publicly identified and should be empowered to answer public enquiries on all matters arising. Letting the Feldberg case come to light has fortified only the lobbyists. □



Lax enforcement of animal rules alleged

■ Contraventions found at MRC institute

■ Home Office inquiry launched

London

ALLEGATIONS of serious misconduct by a senior scientist at the UK Medical Research Council (MRC)'s National Institute for Medical Research (NIMR) in London have brought into question the effectiveness with which Britain's rules on animal experimentation, substantially reformed in 1986, are being enforced.

A Scottish animal rights group, Advocates for Animals, has submitted to the Home Secretary, David Waddington, a report on experiments done by Professor Wilhelm Feldberg, an 89-year-old researcher, and his assistant, John Stean. The report, which includes videotaped evidence, catalogues a number of alleged contraventions of the rules under which a Home Office licence to perform animal experiments is granted. The dossier of evidence was compiled by two animal rights activists, who obtained invitations to Feldberg's laboratory by deception. One, Melody MacDonald, posed as Feldberg's biographer for two years, while the other, Michael Huskisson, videotaped experiments over five months under the pretence that the tapes would be used to teach US students. The pair approached Advocates for Animals with their evidence last month.

The tapes show rabbits struggling, apparently under only light anaesthesia, while surgical procedures are carried out and their body surfaces are heated to over 130 °C. Feldberg's work concerned the mechanisms by which blood sugar levels become raised after heating. Animals were also left unattended during experiments, according to Les Ward, director of Advocates for Animals.

A Home Office statement says the allegations are taken "very seriously" and that its Animal Procedures Committee will be asked to review the general lessons to be learned from the case in order to determine whether Feldberg's alleged misconduct is an isolated instance or an indication that the 1986 Animals (Scientific Procedures) Act is being poorly implemented.

The 1986 Act reformed legislation which had stood since 1876, and instituted a stricter system of licences for individual procedures, projects and scientists. Its enforcement relies on monitoring by Home Office inspectors and by named people within laboratories who are made responsible for the day-to-day care of experimental animals.

Clive Hollands, a previous director of

Advocates for Animals (then known as the Scottish Society for the Prevention of Vivisection), advised the government during the drafting of the act. His participation angered most other factions within the animal rights movement. Ward says that Advocates for Animals made their allegations public to draw attention to the failure to enforce the act, and adds that the group feels that the trust it showed by supporting the act has been betrayed.

Michael Balls, from the Fund for the Replacement of Animals in Medical Experiments, also advised the government on the 1986 Act. He says his commitment to the act is unaffected by the allegations. But he acknowledges that questions must now be raised concerning the "universality" of its enforcement. There may well be other individuals in violation of their licences, Balls says, but he adds that "I don't believe their number is great".

Balls believes that there are enough Home Office inspectors to enforce the act (currently 20, with nearly 18,000 licence holders). Animal researchers are "people with integrity", he says, and any infringements of the 1986 Act are usually the result of misinterpretation, rather than "wilful attempts to avoid the law". But one biomedical scientist, who wished to remain anonymous, questioned whether some inspectors have sufficient understanding of modern scientific procedures.

Whatever the general lessons to be

learned from the case, an inquiry is to be launched into arrangements at NIMR. The roles of senior NIMR staff, Home Office inspectors, the day-to-day animal care officer and the veterinarian responsible for regular checks on the laboratory will come under scrutiny. Balls says that other scientists at NIMR had a "moral responsibility to protect [Feldberg] from himself". In the videotapes, Feldberg is seen as a frail old man, whose eyesight, by his own admission, is failing him. There is concern that Feldberg's eminence — he is a fellow of the Royal Society and was recently awarded a medal by the British Pharmacological Society — may have exempted him from proper monitoring.

Staff at NIMR have been instructed to refer questions to the MRC's head office. The MRC says that its investigation will include a review of procedures for monitoring the continued competence of researchers to work on animals. Dai Rees, MRC secretary and a former director of NIMR, adds that the MRC inquiry will not be a solely internal affair: "credible independent opinion" will be consulted.

The MRC supported Feldberg's work up to 30 April this year, but had decided to discontinue his grant on 6 March. After hearing this, Feldberg is believed to have asked for permission to continue his work without an MRC stipend. Nick Winterton, head of MRC's secretariat, says that NIMR director John Skehel was already intending to wind the work up, but acknowledges that this process may have been accelerated by the allegations. Feldberg and Stean, who is also over normal retirement age, returned their licences to the Home Office the day after the Advocates for Animals evidence had been brought to the attention of the Home Office.

Peter Aldhous

WEST GERMANY

Paul Ehrlich Institute opened in Langen

Munich

RESEARCHERS from the United States, Israel and Europe converged on Langen, West Germany, two weeks ago to celebrate the official opening of the new Paul Ehrlich

The building, which has a shape reminiscent of an antibody molecule, has taken six years and DM335 million (\$200 million) to construct. It is still not complete. PEI president Reinhard Kurth hopes to bring

all 320 employees, including 70 scientific staff members, from the cramped quarters in Frankfurt to suburban Langen by the end of 1990.

PEI was established in Frankfurt in 1899 as the "State Institute for Experimental Therapy" by and for Paul Ehrlich (1854–1915), who was one of the founders of modern immunology and originator of chemotherapy. Ehrlich, a German Jewish physician from Breslau, Silesia, achieved recognition for inventing

Salvarsan, an arsenic-based drug that was the first cure for syphilis. He won the Nobel prize in 1908. Steven Dickman



Institute (PEI) which serves both as a research centre and the federal office for sera and vaccines.

SPACE

Privatization for Japan

Tokyo

JAPAN is about to take its first step towards privatizing its space industry. On 5 July, a huge private consortium will set up a company to mass-produce Japan's next-generation rocket, the H-II, from 1994.

Establishment of the new company comes close on the heels of an agreement with the United States that Japan's government-run National Space Development Agency (NASDA) will launch only research satellites, and that all commercial satellite building and launching will be opened up to competitive, including foreign, bidding (see *Nature* 344, 578; 12 April 1990).

Although no official statement has been made, it seems likely that the reason for transferring responsibility for H-II production from NASDA to the private sector is ultimately to allow commercial launches using the H-II. But there are many barriers to the establishment of such a commercial launch operation.

The new company is being set up with an initial target investment of ¥480 million (\$3 million) by more than 70 companies, including Mitsubishi Heavy Industries, Ishikawajima-Harima Heavy Industries, Kawasaki Heavy Industries, Nissan, NEC, Toshiba, Fujitsu, Mitsubishi Electric, Japan Aviation Electronics Industry and a number of insurance companies interested in insuring satellites.

At present, the Science and Technology Agency (STA), to which NASDA is affiliated, orders rockets one at a time from private industry, which means that even prime contractors such as Mitsubishi Heavy Industries and Nissan cannot risk setting up a production line and building up large inventories of spare parts. NASDA rockets are thus much more expensive than their equivalents in the United States and Europe.

After two test launches of the H-II by NASDA in 1993, the new company will take over ordering, production and delivery of the rockets. Production costs are expected to be considerably reduced and, as pointed out in a NASDA document describing the new company, the next logical step after reducing costs would be for the company to take over launch of the rockets. But "at present" that is not a goal, according to the document.

Katsuo Yonezawa of NASDA's international affairs division says there are many legal and political hurdles to overcome before a commercial launch business can be established in Japan. The law governing NASDA's operations limits the agency to research and development, and was written that way because of an agreement with the United States for the transfer of US rocket technology to

NASDA — NASDA's early N-series rockets were based entirely on US rocket technology.

The recent dispute with the United States over satellites arose because Japan tried to claim that next-generation communication, broadcasting and meteorological satellites which NASDA planned to launch in the mid-1990s would be for research and development purposes only. But under the new agreement, Japan accepts that they are "commercial" and thus under the NASDA law they cannot be launched by NASDA.

The law would have to be rewritten if the new private company were to use NASDA's launch facilities for commercial purposes. But Yonezawa says there are no moves afoot to change the law, particularly under the present political climate where the United States has put strong pressure on Japan to open up its commercial satellite market to foreign countries.

Another barrier has been erected by the fishermen of Tanegashima island where NASDA's launch centre is located. Under an agreement with the fishermen, NASDA is limited to two launches a year during two 45-day periods in summer and winter. In return, the fishermen get several million dollars compensation every year for supposed damage to tuna fisheries.

The new launch facility for the H-II at Tanegashima will be capable of launching four rockets during the two 45-day periods because one rocket can be kept on standby while another is on the launch pad. But the two extra launches will require renegotiations with the fishermen, Yonezawa says.

But even four launches a year will probably not be enough for a commercial operation. Dieter Brand of the Tokyo office of ArianeSpace, the world's first commercial rocket launching company, says that about eight launches per year carrying two satellites are needed for a successful commercial operation (ArianeSpace is capable of ten such launches a year).

Even if all these hurdles are overcome, there is still likely to be strong opposition from the United States. By 1994, the Japanese government will have spent ¥237,000 million (\$1,500 million) on development of the H-II and another ¥96,000 million on new launch facilities for the rocket.

Any attempt to hand these assets over to a private company competing in the international market for commercial satellite launches is likely to raise complaints from the United States that the Japanese government is unfairly subsidizing a commercial operation.

David Swinbanks

NATURAL HISTORY MUSEUM

Another strike

London

RESEARCHERS at the Natural History Museum in London went on a second one-day strike last Friday (11 May) over the museum's controversial corporate plan (see *Nature* 345, 99; 10 May 1990). The researchers, who belong to the Institution of Professionals, Managers and Specialists (IPMS) were protesting about the loss of one in six research posts under the plan. The IPMS claims that it was not consulted during the plan's draft stages. This second strike follows the failure of discussions last Wednesday (9 May) between IPMS representatives, the museum's director, Neil Chalmers, and the museum's board of trustees, about how the plan might be amended to take more account of the researchers' interests.

In the meantime, Sir Hugh Leggatt, a member of the 14-strong Museums and Galleries' Commission (MGC) had received so many letters expressing concern at the NHM's corporate plan that he went to the museum to assess the situation for himself. The MGC is an independent body of distinguished academics that advises both government and museums on matters of policy. Although Leggatt notes "a very serious sense of grievance on the Union side", he recognizes the constraints imposed on management faced with a decrease in core funding: "the basic problem is — in one word — money".

■ See also Correspondence, page 198. □
Henry Gee

Correction: Professor Nicolae Simionescu



INEXCUSABLY, for an international journal on an international subject, the caption beneath the photograph of Professor Nicolae Simionescu of the Institute of Cellular Biology and Pathology in Bucharest (*Nature* 344, 614; 1990) included a misleading English catchphrase. In Britain, to "help the police with the inquiries" is the euphemism describing those suspected of crimes who are questioned but who have not yet been charged. In Romania, to "help the Securitate with their inquiries" understandably means something different. *Nature* apologizes to Professor Simionescu and his colleagues for this unfortunate phrase. □

Memorial ceremony to be held

Munich

THE West German Max Planck Society (MPS) last week set 25 May as the date for a memorial ceremony to Nazi euthanasia victims whose brains had been used for research purposes by the Kaiser Wilhelm Society, the forerunner of MPS. Officials from the local Jewish community as well as Catholics and Protestants will be invited to take part, said MPS official Peter Gutjahr-Löser, although Jews were not believed to have been among the victims.

MPS last year removed thousands of brain tissue samples from its collections at three institutes in Frankfurt, Cologne and Munich and quietly buried them at a cemetery in Munich in February. Some of the samples belonged to the infamous Hallervorden collection (see *Nature* 339, 498; 1989).

Some scientists in Canada and the United States earlier this month called for a worldwide moment of silence and the broad participation of medical researchers in a ceremony commemorating the victims, but MPS chose a quieter route because it did not feel that it alone should be publicly associated with crimes that took place during the Nazi era. Gutjahr-Löser supported the suggestion by Wolf Singer, director of the Max Planck Institute for Brain Research in Frankfurt, that researchers' complicity in such crimes should instead be discussed at a workshop of the International Brain Research Organization, of which Singer is a member.

Even the memorial ceremony has been kept quiet in West Germany, says Gutjahr-Löser, in order to avoid disruptions and allow the ceremony to proceed in a "dignified" manner. Other public occasions with a connection to the Nazi period, such as the 1987 funeral of Nazi war criminal Rudolf Hess, drew much unwanted interest from neo-Nazi groups. The time and place of the Hess funeral was kept secret, but this did not prevent neo-Nazi outbursts at the place where he was expected to be buried.

The samples would have been buried and the ceremony held sooner, said Gutjahr-Löser, but a Frankfurt university professor involved in the decision (who is not a member of MPS) questioned the propriety of burying samples whose origins were unknown. Nevertheless, MPS decided to bury any sample where there was any question of its having derived from a euthanasia victim.

The text of the inscription carved on the stone at the memorial will read: "In remembrance of the victims of National Socialism and their abuse by medical science. To all researchers an exhortation to set their own boundaries in a responsible manner."

Steven Dickman

US to end modernization

Boston

THE Bush administration has offered to end the United States' chemical weapons modernization programme in exchange for several Soviet concessions, according to officials inside and outside the Bush administration. The possible reversal of a programme that the administration had supported steadfastly was uncovered by the press last week, but is said to have been proposed during Soviet Foreign Minister Eduard A. Shevardnadze's visit to Washington last month.

State Department officials formally acknowledge only that a "range of possibilities" concerning chemical weapons are being discussed with the Soviets. But a number of inside observers express confidence that a bilateral agreement will come soon, perhaps during the summit between Presidents George Bush and Mikhail Gorbachev planned for the end of this month in Washington. In this agreement, they say, the United States is likely to abandon its programme to modernize binary chemical weapons. Binary weapons, which produce a lethal substance only when two separately stored chemicals are mixed, are more stable in storage than the older poison-gas weapons.

In particular, the United States is said to have offered to stop production of new binary chemical weapons in exchange for a firm and speedy timetable for the destruction of current chemical stocks and an agreement on the contentious issue of

whether the United States can, as the administration desires, retain two per cent of its chemical weapons stockpile as a deterrent against nations that do not sign a global treaty banning the weapons. The Soviets have maintained until now that no such stockpiles should be allowed.

Critics of the administration say that the move to abandon the production of binary weapons has come only because of technical and political problems that are besetting the modernization programme. Recently, for instance, two US-based companies refused to provide an essential ingredient for one of the proposed chemical weapons (see *Nature* 344, 802; 26 April 1990). In this sense, critics say, Bush is merely trying to turn a faltering programme into a diplomatic bargaining counter.

Elisa Harris, a senior research analyst at the Brookings Institution in Washington, admits that the beleaguered state of the modernization programme "may have helped to tip the balance" toward the latest US proposal, but argues that the administration "deserves credit" for taking stock of a changed situation and a substantially changed "chemical threat" from the Warsaw pact.

Harris and others say that a bilateral US-Soviet agreement will give an important boost to multilateral talks and "remove lingering doubts in Geneva" about the US commitment to a global ban on chemical weapons. **Seth Shulman**

BIOLOGICAL WEAPONS

International treaty made domestic law

Boston

FIFTEEN years after the US Senate ratified the Biological Weapons Convention of 1972, an international treaty that bans the development, stockpiling and use of biological weapons, the Congress finally approved last week a bill that renders the provisions of the international treaty into domestic law. The bill prohibits any individual from knowingly producing or possessing any biological agent for use as a weapon. As in the international treaty, biological research on toxins or biological agents that is conducted for peaceful purposes is exempted.

Although the bill was approved unanimously, its passage comes only after several failed attempts over the past few years. The new legislation is seen as a victory for scientists and officials who have denounced stepped-up military interest, in the United States and abroad, in biological agents. In response to a fourfold increase in US military support for biological research, more than a thousand biomedical researchers have pledged in the past two

years not to engage knowingly in research that would further the development of chemical or biological arms (see *Nature* 334, 279; 1988). Many of these researchers have argued that the United States' failure to adopt a domestic law specifically banning the production of biological weapons undercut its formal support for the treaty.

Jonathan King, a biologist at the Massachusetts Institute of Technology who sponsored the pledge campaign on behalf of the Council for Responsible Genetics, called the legislation's passage "a very important step" in "ensuring that biotechnology is developed for peaceful means" and says it provides an important "enforcement tool" against research that may violate "the spirit, if not the letter" of the treaty. He also says that the legislation's timing is important because a treaty review conference is scheduled for next year. The congressional action, he says, may help build a climate in which signatory nations can work for strengthened treaty verification measures. **Seth Shulman**

ENVIRONMENTAL CONFERENCES

US and UK to block limits

London & Washington

A row is likely to blow up at an international conference on sustainable economic development in Bergen, Norway, over the wording of a ministerial declaration to be produced on 16 May. Several nations, led by the United States, object to Norwegian proposals to set definite targets for environmental improvements, notably a commitment to stabilize carbon dioxide emissions at today's levels by the year 2000.

The Bergen conference is organized by the United Nations Economic Commission for Europe (UNECE), and is one of four regional conferences following on from the 1987 report of the United Nations World Commission on Environment and Development, chaired by Gro Harlem Brundtland, then prime minister of Norway. The four regional conferences will assemble advice in preparation for a global conference on the environment and development, planned for 1992.

The environmentalist pressure group Friends of the Earth released a leaked telex, dated 24 April, which they say was sent from the US government to a number of its embassies. The telex says that US officials expect Britain, Canada and the Soviet Union to agree with the US position that no specific targets for carbon dioxide emissions should be adopted at least until the release, later this summer, of reports from the three working parties of the Intergovernmental Panel on Climate Change. These reports will be debated at a World Climate Conference in the autumn. The leaked telex advises US officials abroad that West Germany, the Nether-

lands, Belgium, France, Denmark and Sweden will support the Norwegian advocacy of rigid targets.

Although the content of the telex is unsurprising, as it repeats publicly stated policy, its release is likely to add to environmentalists' belief that the US and UK governments are not giving environmental issues urgent consideration. Misgivings have also arisen over these countries' choice of delegates for the conference. In both cases, top-level cabinet ministers have been replaced by junior officials. Britain is represented by David Trippier, deputy to Secretary of State for the Environment Chris Patten, who begged off on the grounds of domestic work pressures. And although US Environmental Protection Agency administrator William Reilly had been invited to the conference, the White House decided instead to send a small team headed by John Knauss, a scientist from the National Oceanic and Atmospheric Administration.

In the United States, where leaked government documents and internal policy disputes are becoming a familiar feature of environmental negotiations, environmentalists are pointing to the latest controversy as a further indication that the Bush administration has underestimated the ability of the environment to dominate international politics.

"Until the administration realizes that the environment is a powerful issue, they'll have to be dragged kicking and screaming into more action," says Michael Oppenheimer of the Environmental Defense Fund.

**Peter Aldhous
& G. Christopher Anderson**

US says no to international ozone plan

Washington

ENVIRONMENTAL ministers at a United Nations conference in Geneva sharply criticized the United States last week for opposing a plan to help developing countries to phase out ozone-destroying chlorofluorocarbons (CFCs) and use less damaging but more expensive alternatives. Of the 50 or so countries represented, only the United States rejected the plan, which would set up a \$100 million fund to help developing countries switch to CFC substitutes as they become available. US officials said that they believe the World Bank is the best source for such a fund.

Delegates were meeting in Geneva to prepare for a conference in London next month at which the 54 nations that have ratified the Montreal Protocol on CFC reduction will discuss strengthening the treaty. At present, the protocol calls for a 50 per cent worldwide reduction in CFCs by the year 2000, but, with a number of

workable CFC alternatives already emerging, many environmentalists have called for a faster phase-out. Many industrialized countries have agreed to go beyond the Montreal Protocol domestically, but developing countries such as China and India have refused to sign the treaty, claiming that they need cheap CFCs — used in refrigerators, for example — to raise their standards of living.

Unless developing countries are given an incentive to abandon CFCs, cuts by developed countries will not be enough to save the ozone layer, delegates at the Geneva conference argued. White House spokesman Roman Popadiuk said that "we want to assist less developed countries, [but] we simply believe that use of existing, established institutions is a more appropriate course". The World Bank, claiming that its money is committed elsewhere, distanced itself from the US proposal.

G. Christopher Anderson

ATMOSPHERIC RESEARCH

While you're up there...

Washington

WHEN two adventurers and a Soviet cosmonaut embark on a round-the-world balloon voyage near the end of the year, they will be carrying instruments that may provide unique data on the ozone layer and atmospheric turbulence, officials said at a press conference held last week to announce the project. As part of an agreement worked out with the US National Aeronautics and Space Administration (NASA), the three-man balloon will carry scientific instruments in exchange for navigation and communications assistance from the space agency.

Richard Branson, the British entrepreneur who founded Virgin Records and Virgin Atlantic Airways, is underwriting most of the project and will be the balloon's captain. Larry Newman, who gained the current balloon distance record with a trans-Pacific voyage in 1981, and Vladimir Dzhanibekov, a Soviet cosmonaut, will complete the crew.

If winds, weather and equipment cooperate, the balloon, known as Earthwinds, will be the first to circumnavigate the globe. It will be launched from a former dirigible hangar in Akron, Ohio, some time between November and February. With jetstream winds that can reach 200 m.p.h., project planners hope the trip will last less than three weeks.

NASA instruments on board the 24-foot gondola will include five accelerometers to measure changes in the balloon's velocity due to wind shear. Vortices in the jetstream can keep water vapour, particles and gases in suspension for long periods and are an important element in atmospheric chemistry. But few vortex observations have extended beyond relatively small geographic areas.

Earthwinds will try to follow the 40th parallel all around the world, and should provide a complete global strip of atmospheric turbulence data, NASA scientists say. Other instruments will include two anemometers to measure wind shear beneath the balloon and radiosonde packages to measure temperature, pressure and humidity.

NASA scientists also hope to gain a new global perspective on the ozone layer with a radiometer that will measure the intensity of ultraviolet light shining on the balloon from above. Because the balloon, at an altitude of about 35,000 feet, will be at the bottom of the stratosphere, the amount of ultraviolet light received should measure the amount of stratospheric ozone above the balloon's path.

Beyond the direct scientific value of such information, NASA hopes to use the data to calibrate its orbiting Total Ozone Mapping Spectrometer, which has been aboard the Nimbus 7 satellite since 1977.

G. Christopher Anderson

Wellcome drops TPA

Washington

THE winds of fortune shifted in Genentech's favour last week when the UK-based Wellcome Foundation announced that it is abandoning a six-year research effort into the blood-clot dissolving agent tissue plasminogen activator (TPA), together with studies into novel plasminogen activator (NPA), a 'second-generation' TPA.

Wellcome's decision leaves Genentech, whose Activase is the only TPA drug approved for sale in the United States, with a clear monopoly. Although it faces competition from Hoechst-Roussel Pharmaceuticals' streptokinase and SmithKline Beecham's recently approved Eminase, two thrombolytic drugs unrelated to TPA, Activase has two-thirds of the US market with 1989 sales of \$196.6 million.

Wellcome had filed with the US Food and Drug Administration for approval of its version of TPA, but a month ago the jury in a district court in Delaware ruled that both TPA and NPA, which are licensed to Wellcome by Genetics Institute, infringed Genentech's US patent rights (see *Nature* 344, 692; 19 April 1990).

Wellcome spokesman Martin Sherwood said the company's decision "was made on a number of grounds", citing a recently published Italian clinical trial that questioned TPA's therapeutic benefit over streptokinase, which is one-tenth of the price (see *Nature* 344, 183; 15 March 1990), as well as the patent ruling. The company chose not to wait for the preliminary results of the Oxford-based ISIS-III clinical trial, expected later this year, which will provide the first direct comparison of Wellcome's TPA, Eminase and streptokinase.

In what amounts to a strategic shift of resources, Wellcome announced that it is 'stepping up' development of its interferon product, Wellferon, for the treatment of hepatitis B. Wellcome also reaffirmed its commitment to WelGen, a joint mammalian cell production facility that it is developing in conjunction with Genetics Institute. Wellcome hopes to manufacture Wellferon at WelGen when the unit becomes operational in 1991.

When Wellcome entered into the licensing agreement with Genetics Institute, it was thought that TPA might achieve worldwide sales of \$1,000 million, making it the first blockbuster biotechnology drug. But sales have been disappointing, and according to UK stock analyst Ian Smith it will achieve its early promise only if other medical uses are found. Financially, Smith says, TPA was "only of marginal significance to Wellcome". But stock analyst Peter Drake differs: estimating that only 20–25 per cent of the throm-

bolytic market in the United States has been penetrated, he believes that Wellcome "made this decision based on the patent problem and not on the ultimate size of the TPA market".

Several other companies, including Eli Lilly, Upjohn, American Home Products, Wyeth and Boehringer Mannheim, are thought to be working on second-generation TPAs, but may now have to review

AIDS CONFERENCE

French not to attend

Paris

THERE will be no official French representation at the Sixth International Conference on AIDS in San Francisco in June because of US Immigration and Naturalization Service rules that anyone testing positive for infection with HIV (human immunodeficiency virus) may be denied entry. French Health Minister Claude Evin told the 43rd World Health Congress in Geneva last week that despite the "good will" of the US authorities, the creation of a special visa for HIV seropositive travellers still does not go far enough.

Although French delegates wishing to attend the conference are not affected by this official stance, the French National Agency for the Fight Against AIDS has arranged for satellite transmission of plenary sessions to be screened in a 900-seat hall at the Paris Science and Technology Museum at La Villette.

Peter Coles

BSE

Spongiform encephalopathy found in cat

London

A POST-mortem diagnosis of spongiform encephalopathy in a British cat has provoked new concern that Bovine Spongiform Encephalopathy (BSE) could be transferred to the human population. The case, in a five-year-old Siamese male, is the first time a spongiform encephalopathy has been found in a cat. But other cases may have gone undetected, being dismissed as general neurological disorders; spongiform encephalopathies can be identified only after death by the characteristic microscopic holes found in infected brain tissue.

Agriculture ministry chief veterinary officer Keith Meldrum says that there is no evidence for a connection with similar diseases in other animals, but speculation is rife that the animal may have contracted the disease by eating pet food containing cattle or sheep tissue infected with spongiform encephalopathy.

The new suggestion of cross-species transmission of spongiform encephalo-

pathy has renewed calls for the government to tighten up animal feed regulations, to prevent infective agents entering the human food chain. David Clark, Labour agriculture spokesman, wants a ban on the feeding of sheep and cattle offal to pigs and poultry. So far, research has suggested that these animals are not susceptible to the diseases. The agriculture ministry is to commission research to see if the cat spongiform encephalopathy can be passed to laboratory mice. This should reveal if the cat infection behaves similarly to BSE and scrapie (a similar disease of sheep, thought to be caused by the same infective agent as BSE), which have both been passed to mice.

Genetics Institute and Wellcome have filed for a reversal of the jury's verdict by the judge, but a ruling is not expected until September.

Diane Gershon

UK NUCLEAR INDUSTRY

New AEA chairman

London

JOHN Maltby, currently chairman of Burmah Oil, is to take over as chairman of the UK Atomic Energy Authority (AEA) from 1 July.

The appointment will allow the present chairman, John Collier, to concentrate on running Nuclear Electric, the largest of the two new state-owned nuclear electricity generating companies formed earlier this year as a prelude to privatization of the non-nuclear electricity generating industry.

■ The AEA has also announced the 1992 closure of its prototype pressure tube boiling water reactor at Winfrith, Dorset. The decision is part of a series of cuts at AEA, forced by the current hiatus in Britain's nuclear programme. The Institution of Professionals, Managers and Specialists, a union which represents scientists and engineers at Winfrith, blames the government's electricity privatization plans for the reactor's closure.

Peter Aldhous

Scientists given the jitters

Washington & Tokyo

THE US Department of Defense (DoD), whose network of navigation satellites has recently allowed scientists to determine geographical positions with unprecedented accuracy, has begun intentionally degrading the signals so that potential military adversaries are unable to take advantage of the system. But researchers in Japan and elsewhere say that the real victims of this misinformation could be scientists trying to forecast earthquakes by using the satellites to detect minute precursor ground motions.

The Global Positioning System (GPS), a constellation of two dozen satellites that broadcast signals to ground-based receivers, has been in place since the late 1970s. Each satellite broadcasts a signal that specifies its position and the relative positions of receivers on the ground. Using the signals from several satellites, aircraft, ships and other vehicles can find their position by triangulation to within 40 metres.

But in recent years, researchers have begun averaging many readings over time to find locations with errors of as little as one centimetre (*Nature* 343, 590 & 631, 15 February 1990). This accuracy makes GPS measurements useful for seismology: movements in the Earth's crust in the weeks before an earthquake can translate into positional changes of several centimetres, and by monitoring the relative positions of GPS receivers that have been placed around likely trouble spots, researchers may be able to predict impending earthquakes by watching for unexpected changes in positions.

When the GPS system was first launched in 1978, these sorts of precise scientific applications were completely unanticipated. But although the usefulness of the first 'block' of GPS satellites proved to be an unexpected windfall for scientists and other users, the second block of satellites now going up has even greater potential. Indeed, from the beginning, the DoD considered the second generation of GPS satellites too accurate for 'unauthorized' use.

Built into the second-generation GPS satellites are electronics that slightly alter the transmitted signal to cloak the true position of the satellite. 'Authorized users' have been given equipment that can unscramble the distortion to reveal a satellite's exact position at any time. Known as 'selective availability' (SA), this method has been in use for about a month.

For unauthorized users — a group that includes most scientists — the effect of SA is that signals that could be trusted to provide 1 cm accuracy a month ago are now only good to about 6 cm. Although

SA has been planned for years and the DoD issued warning notices several months ago, many researchers were nevertheless caught off-guard by the sudden signal degradation in March.

US researchers are appealing to GPS officials to reverse the policy. The American Congress on Surveying and Mapping is asking the DoD to treat SA as a trial programme, and argue that researchers should have access to the full GPS accuracy in times of peace, saving SA for outbreaks of hostilities. But until the DoD reconsiders SA, the best solution, suggests one researcher, is to "write [to] your congressman". DoD officials are considering several alternatives that may appease the scientific community. One solution would be to allow researchers to apply for authorized-user status, so that they could be given access to special decoding equipment. But it is not clear what criteria would be taken into consideration when judging such applications.

Scientists may find that more readings over a longer period of time will give full GPS accuracy, officials say. Or research teams can take data they know are somewhat incorrect, then wait several weeks

SCIENTIFIC MISCONDUCT

Dingell tries again

Washington

Representative John Dingell (Democrat, Michigan) tried once again, last Monday, 14 May, to establish with the help of forensic evidence from the Secret Service that part of the data in a 1986 *Cell* paper by David Baltimore, Thereza Imanishi-Kari and others was fabricated. As in a previous hearing last year (see *Nature* 339, 163; 18 May 1989), Dingell argued that Imanishi-Kari had altered her laboratory notebooks to conceal the fact that key experiments in the paper had never been carried out.

But Imanishi-Kari, who refused to appear at the hearing and made her rebuttal at a press conference, responded, as she had done last time, that her only sin was poor note-keeping. Dingell says he intends to turn the evidence over to the Justice Department for possible criminal prosecution.

At Monday's hearing, before Dingell's oversight and investigations subcommittee, two Secret Service agents recounted their analysis of paper tapes from gamma-ray counters in Imanishi-Kari's laboratory at the Massachusetts Institute of Technology. From examination of the typeface of the printer and the wear on the ink ribbons, they concluded that the order in which the tapes appear in the notebooks is not the order in which they were pro-

duced, and that the tapes were not produced on the dates marked on the corresponding notebook pages.

The agents argued that many of the data may have been created after allegations of misconduct first arose in the spring of 1986. But because there is no independent record of when the experiments were actually done, inconsistency between various dates is all the Secret Service has been able to prove.

Imanishi-Kari said last week that she has never disputed that dates in her notebooks may not correspond with experiments. She said she sometimes transcribed experimental notes into the notebooks months after the experiments were over. The dates "could mean the day the experiment began, or the day it finished. If I had a better record I would put it forward. But that is how I take notes."

Suzanne Hadley, former director of the National Institutes of Health (NIH) office of scientific integrity, told the hearing that NIH have cut off funds for one of two grants to Imanishi-Kari because of "significant questions about her ability to hold a grant". But she added that that does not imply that NIH has determined misconduct; improper record-keeping is also a grant-losing offence.

G. Christopher Anderson

& David Swinbanks

G. Christopher Anderson

Opportunities for the East

Who is doing what to help Eastern European scientists? And where can an Eastern European scientist look for a new source of funds? What follows provides a guide. But established researchers in Eastern Europe should be aware that the first step is still almost always to find a colleague in the West willing to help support a grant application.

Europe

European Communities (EC). Future East European involvement in the EC's Framework research programme is being debated, but a decision is not expected for some months. Poland and Hungary may be the first to join, having signed agreements for cooperation in science and technology.

In higher education, TEMPUS, a counterpart to the EC's ERASMUS scheme for the mobility of students between EC member states, is to be approved this week. It will at first include only Poland and Hungary, but is expected to expand to cover the whole of Eastern Europe. The scheme will allow students to travel from East European universities to work at EC universities or in industry for three months to a year. There will also be schemes for university staff to move between East European and EC institutions, and to set up projects linking EC and East European universities. But these are aimed at improving training, and are not research based.

The **European Science Foundation (ESF)**, based in Strasbourg, has members which are public grant-making agencies from EC countries and elsewhere. Hungarian membership will be considered later this year. But individuals from countries not represented may (and do) take part in research networks in fields as different as personal development and polar science.

Scientists from countries not represented by ESF membership also take part in some of the more formal collaborative programmes organized within ESF; at least some local costs arise. The European Training Programme in Brain and Behaviour Research has a series of workshops and offers 30 short-term (up to 3 months) fellowships for people younger than 35 as well as travel grants to meetings. There are also small grants for essentially collaborative research between established neuroscientists in the same field. ESF also provides short-term visiting fellowships (up to two months) in toxicology.

The **International Union against Cancer (UICC)**, based in Geneva, runs a number of schemes for cancer researchers to work abroad, or to set up joint research projects between laboratories in different countries. Young Eastern European molecular

biologists may apply to work in other European countries through the **European Molecular Biology Organization (EMBO)**'s long-term and, exceptionally, short-term fellowships. Like the ESF, UICC and EMBO do not run schemes specific to Eastern Europe.

France

The French **Ministries for Research and Technology (MRT)**, for **Foreign Affairs** and for **Education** have just announced measures to improve research exchanges between French laboratories and researchers in Eastern Europe.

Until now, arrangements have been on an *ad hoc* basis, between senior researchers. Countries in Eastern Europe were not treated any differently from other countries. This usually meant that few contacts could be established. In 1989, about 250 man-months were paid for in grants by the Ministry for Foreign Affairs for senior researcher exchanges.

Since October last year, the ministry says there has been an "exponential increase" in demand. Fifteen high-level fellowships have been awarded to East European researchers this year and the ministry expects 60–70 such fellowships to be awarded this year. Similarly, the **National Centre for Scientific Research (CNRS)** and the **National Institute of Medical and Health Research (INSERM)** have exchange agreements with the relevant academies of most Eastern European countries, except Albania.

The French government last month adopted an "emergency plan" of aid to Eastern European countries which includes measures to improve exchanges and to offer better opportunities for training of young researchers from these countries.

By the end of 1990, MRT hopes to have made 700 grants for senior researchers, young postdoctoral researchers and a number of technicians and engineers to spend between 3 months and 18 months in France. Further expansion will follow. As a rule, awards to researchers are made on the initiative of the French host laboratory and no mechanism yet exists through which Central and East European researchers can apply directly.

In order to "intensify personal contacts" and exchanges with Eastern and Central European countries, the Ministry for Research and Technology has earmarked FF200,000 each for 20 seminars by the end of the year. Scientists from Eastern Europe will also be able to benefit from a FF3 million fund set up to allow them to attend symposia in France this year.

Other measures put forward by MRT include "twinning" of French laboratories with counterparts in Eastern Europe. MRT is also creating 200 "welcome" posts

in French research organizations. These will be valued at FF150,000 each and will allow senior researchers to hold posts for six months, alternating with six months in their home laboratory.

West Germany

As yet there are few grants available for Eastern Europeans to work on independent or joint projects in their own countries.

Except in the case of East Germany, any such grants are regulated by inter-governmental agreements and are arranged for the most part through the academies on the East European side and the **Deutsche Forschungsgemeinschaft (DFG)** and **Research Ministry (BMFT)** on the West German side.

DFG is offering a minimum of DM5 million (\$3 million) for around 100 joint projects carried out by at least one East and one West German partner. DFG is making a limited exception to its practice of not paying for scientific equipment, which in West Germany is supplied by the *Länder* (states). Equipment awarded is nominally delivered to the West German partner, which can pass it on to the East German group.

East Germans at present need to have partners in the West before they can apply for money. But this may soon change and East Germans may be able to apply directly for grants from DFG.

Opportunities are greater for those who would like to travel to West Germany. Researchers may apply for fellowships to the **Humboldt Foundation**. The fellowships are well paid but competitive — just 30 per cent of applicants succeed. The foundation gave 85 fellowships in the natural sciences from six Eastern European countries in 1988.

Researchers and professors who would like to spend one to three months at a West German institution or to attend a scientific conference in West Germany can apply to the **German Academic Exchange Service** (Deutsche Akademische Austauschdienst or DAAD). In 1988, DAAD helped to pay for over 1,000 Eastern European researchers to attend conferences in all fields of humanities and natural science. Students and postdoctoral researchers may apply for one-year grants to work in West Germany.

Last year, the **Volkswagen Foundation** set up a programme for East German research groups. The foundation offers as much as DM10 million in direct aid to East German researchers and applications have already begun to flow.

Bonn is not the only place to turn for help in West Germany. Several of the West German *Länder* are offering money to East Germans who wish to attend conferences or collaborate with researchers in those *Länder*. **Baden-Württemberg**, for example, is expected to offer about

DM3.5 million in 1990 for "university, scientific and cultural exchange" with researchers in Saxony, especially in the Dresden region. Other *Länder* have programmes for their 'partner regions' in the East.

The Research Ministry has also approved DM80 million in extra funds for East German research (much of it applied research) for 1990. Some of this money may be given to East Germans directly.

United Kingdom

The **Royal Society** has exchange agreements with academies of science in all Eastern European countries (except Albania). Apart from the Bulgarian programme, which is not very active, each agreement is nominally for between 12 and 20 person-months of exchange a year, although the programmes are constantly expanding. Some joint research projects, linking East European and UK laboratories, are also supported.

Eastern European scientists can visit the United Kingdom on short 'study visits' or for longer 'fellowships'. The latter are for young postdoctoral researchers, and are being expanded. East European scientists interested in the Royal Society's exchange programmes should apply through their own academy.

The **British Council's** Cultural Exchange Programmes allow scientists from Bulgaria, Czechoslovakia, East Germany, Hungary and Romania to travel to Britain for short research visits and provides some postgraduate scholarships. Limited funds are also available to foster links between British and Eastern European research groups.

For Poland, the British Council runs an Academic Links Project, financed by the UK Government's 'Know How' Fund. Designed primarily to improve training by linking British and Polish higher education institutions, the scheme includes projects in environmental management and agriculture. Similar schemes for Czechoslovakia and Hungary may be announced soon.

The British Council also administers schemes set up by several other organizations, including the **Leverhulme Trust's** East European visiting fellowships which allow one postdoctoral scientist a year from each of Bulgaria, Czechoslovakia, Hungary, Poland and Yugoslavia to work in the United Kingdom for up to 10 months.

The **Wellcome Trust's** European Programme covers all Eastern European countries. Postdoctoral scientists may visit for one year. Application is through a British sponsor. Wellcome also funds shorter research visits. About 20 Eastern European scientists a year visit the United Kingdom under these two schemes, but there are a larger number of travel grants for experienced biomedical researchers to

attend UK scientific meetings or learn new techniques.

The **Cancer Research Campaign** supports visiting fellows at the Patterson Institute for Cancer Research in Manchester. Fellows can come from any country, but the institute has strong links with Eastern Europe. Short visits are preferred, but the budget for 1990-91 is already spent. Experimental cancer researchers interested in visiting the United Kingdom the following year should contact Professor David Harnden but should be aware that competition is severe.

Young Eastern European Scientists should also look out for advertisements (usually in *Nature*) for bursaries to attend the **Ciba Foundation's** London symposia. There are about eight symposia a year, on topics in medical and chemical research, and bursars may work in a participant's laboratory for four weeks.

Austria

The **Ministry of Science** has received 160 million schilling (about \$14 million) for scientific cooperation with Eastern Europe in 1990. Part of the money will be used to invite professors from Eastern European countries.

Austria has eliminated fees for students from Hungary and Romania (Czech and Slovak students were always exempt). The **Technical University** in Vienna plans to create an East-West centre to assist in the transfer of technology.

Austria would like to serve as a liaison for research groups seeking access to large research establishments in Western Europe but the ministry has not yet released details of how this will work.

The ministry says researchers should first turn to the Austrian cultural institutes or embassies in their countries, as well as to their countries' own culture ministries, to get more information about exchanges.

United States

Attitudes towards the old Eastern bloc are changing rapidly in the United States. This month, the **National Institutes of Health (NIH)** are considering a grant proposal that has a Moscow-based Soviet scientist as one of its co-principal investigators. If Maxim Frank-Kaminetskii of the Institute of Molecular Genetics in Moscow and Valery Soyfer of Ohio State University are successful, NIH will have given their first direct grant to a scientist in the Soviet Union.

Bigger changes may be on the way, although the slow pace of the US budget cycle still puts the United States far behind West Germany and France in new initiatives to help Eastern European scientists.

Virtually the only new source of money so far approved comes in the Support for East European Democracies (SEED) Act. The act is intended to help Eastern European countries to establish market

economies but also provides for several major environmental protection schemes.

Most NIH support for foreign visitors involves the **Fogarty International Center**. About 170 Eastern European scientists are helped each year. The number is expected to grow as Eastern Europeans develop the contacts needed to secure invitations to work in the United States. The Fogarty Center also runs a postdoctoral fellowship programme and a small two-way exchange programme with Poland, Bulgaria, Romania and Czechoslovakia. Congress may this year approve a major budget expansion that would also provide the first NIH support for collaborative research in Eastern European laboratories.

The **National Science Foundation** has formal agreements with most East European countries to support cooperative research projects. Each side pays for the expenses incurred in its own country with NSF allowing a total budget of around \$600,000 a year. Interest in the programmes is running very high and there are hopes for a big increase in next year's budget. NSF is also helping East European countries to establish their own peer-review systems.

The **National Academy of Sciences** has exchange agreements with each of the East European academies which provide for a certain number of person-months of exchange each year, ranging from 10 in the case of East Germany to 27 for Czechoslovakia. Applications are made through the foreign academies.

The **Fulbright Scholar Program** is probably the best-known US exchange programme, but there are scholarships for only a handful of scientists each year. Information can be obtained at US embassies abroad.

Hungarian scientists can obtain special help from the **Soros Foundation**. Set up by George Soros, a wealthy expatriate Hungarian, it offers some 50-100 scholarships and student grants for study abroad through its office in Budapest. □

Selected addresses (addresses are not given where the information is better obtained through a local embassy, academy, or a foreign partner.)

United Kingdom. Wellcome Trust, 1 Park Square West, London NW1 4LJ □ Professor David Harnden, Patterson Institute for Cancer Research, Christie Hospital, Manchester M20 9BX □ Ciba Foundation, 41 Portland Place, London W1N 4BN.

Belgium. TEMPUS Office, European Commission, Rue de la Loi 200, 1049 Brussels, Belgium.

France. European Science Foundation, 1 Quai Lezay Marnes-la-Mairie, 67000 Strasbourg.

Switzerland. UICC, 3 Rue du Consol-General, 1205 Geneva, Geneva.

West Germany. EMBO, 6900 Heidelberg 1, Postfach 1022.40 □ Deutsche Forschungsgemeinschaft (DFG), Kennedyallee 40, (Postfach 20 50 04), Bonn 2 □ Alexander von Humboldt Foundation, Jean-Paul-Str. 12, D-5300 Bonn 2 □ Deutsche Akademische Austauschdienst (DAAD), Kennedyallee 50, D-5300 Bonn 2 □ Volkswagen Foundation, Kastanienallee 35, (Postfach 81 05 09), D-3000 81.

Austria. Austrian Ministry of Science, Minoritenplatz 5, A-1014 Vienna □ Technical University of Vienna, Karlsplatz 13, A-1040 Vienna.

The museum director's view . . .

SIR—Many of this museum's scientists have for years used their taxonomic skills to carry out distinguished research on human diseases such as leishmaniasis and schistosomiasis; others have monitored the effects of air and water pollution through their research on lichens, protozoa and meiofauna. Still others are involved in long-term research programmes of insects in tropical rainforests.

All of these and many other of our scientific staff will have been astonished and insulted by your leading article "A major museum goes populist" (*Nature* 345, 1; 1990) where you suggest that the museum has no expertise in areas of human health or environmental biology. Work in these important areas will continue and will be strongly supported in the future, as will taxonomic research in many

areas of pure research. Your leading article is equally at fault when it suggests that taxonomic and evolutionary work will be restricted to one research programme only: taxonomy must and will continue to pervade all our scientific programmes.

The major question that we must face in the museum, and from which your leading article runs away, is one of scientific priorities. No natural history museum, however great, is able to carry out taxonomic research across the whole range of present-day animal and plant life, together with minerals and fossils. There are simply too many species for this to be possible. The only way forward is for our museums to be selective, and we have chosen to concentrate on those areas of taxonomic research where we already have the greatest strength, and where we

see the greatest potential for the future. We have identified six such areas — biodiversity, environmental quality, mineral resources, agricultural resources, human evolution and human health.

All these programmes are totally dependent upon our scientists' taxonomic skills and several of them will benefit from close collaboration with scientists from other disciplines in other institutions.

The need to focus our research is made all the more acute by our funding position. In our corporate plan, we call upon our sponsoring department for additional funding over the next five years. I hope that the many scientists who value our museum, and who recognize it as the unique treasure house that it is, will give us their support as we press our case.

NEIL CHALMERS
Director

*Natural History Museum,
Cromwell Road, London SW7 5BD, UK*

. . . and the views of the onlookers

SIR—You have reported in some detail the corporate plan for 1990–95 of the Natural History Museum. In fact the plan is one of the most blatant pieces of bandwagon-jumping seen in recent years. It almost beggars belief that the whole basis of the research work of one of our greatest scientific institutions can be changed at a stroke, without consultation with users outside the museum, and apparently without adequate consultation inside either (see for example *The Times* of 25 April).

As recently as November 1987, the museum commissioned from the University of Manchester an "Evaluation of the research activities of the British Museum (Natural History)", to which I and many others responded. One aim of this survey was stated to be to "assess the relevance of the Museum's research to other workers in the same, or related, fields". I have not seen the confidential report, but selective summaries (for example in *The Guardian* of 20 April 1988) quoted it as saying that the museum "represents a world class activity" but predicted that the decline in real terms of government funding, and the failure of other sources of revenue to compensate, would cause it to lose this pre-eminence. It also noted that the museum's "ceasing research [of the present kind, implied] would be highly damaging for a large number of researchers outside the museum" (and, it might have added, to other users in government service and in industry).

Yet this is precisely what is now proposed. Instead of research being systematically related to the collections and the animal and plant groups that they represent, it will be diverted to "biodiversity, environmental quality, living resources, mineral resources, human health and human origins". As John Evans recently

pointed out in his presidential address to the Geologists' Association, this means that the museum is abandoning the fields in which it is supreme and unchallengeable for topics some of which are of "ephemeral fashionability", in which it will be in competition with other bodies which are better adapted to such activities.

What is needed, and what the management of the museum has singularly failed to provide, to the evident dismay of many staff, is reaffirmation of the museum's role as a world centre of research in biology. This is not to say that its research must not change. It has changed a great deal since the last century under the anti-darwinian Owen, when identifying, cataloguing and labelling were almost the sole preoccupation of staff. But it must change by evolution, not by *diktat*.

D. T. DONOVAN

*Department of Geological Sciences,
University College London,
Gower Street, London WC1E 6BT, UK*

SIR—The Natural History Museum in London is universally acclaimed as one of the foremost research institutes of natural science worldwide, a remarkable institution with a splendid record of solid contribution.

Those of us concerned with the history, as well as the present and future, of life on this planet regard this institution as an invaluable repository of relevant knowledge, experience, and literally unique and irreplaceable, scientific collections. Until recently, we felt secure in also regarding the museum as a certain source of major advance in the years to come. Henry Gee's note (*Nature* 344, 805; 1990) suggests that because of financial squeeze, with "one of six [science] jobs to go", that

expectation may not be realized.

As one who spent 1989 as a visiting scientist in the museum, I am convinced that Neil Chalmers, the director, is trying to do his best for the institution under exceedingly difficult financial constraints. In fact, it seems to me that far too little credit is given to the director and his staff for the strides the museum has taken towards becoming a more 'user-friendly' sort of place.

The museum has a new image, fine exhibitions, markedly improved public facilities (especially for school children). Moreover, the financial squeeze is by no means limited to the Natural History Museum. What about Kew Gardens? And the V & A? And the venerable British Museum? From my perspective, all seem plagued by the same disease.

But with the financial squeeze in place, who is the loser? The answer is all of us — just as we all benefit from the scientific accomplishments of the museum, we will all lose if those contributions are curtailed. Indeed, in this age of scientific advance, of increased awareness of the importance of environmental quality and of the fragility of our global future, what we need is expansion of the museum's scientific role, not cutbacks.

As a concerned bystander, with a vested interest in the future of the museum, its staff, its scientific prowess, its collections, I can only hope that the Office of Arts and Libraries will accede to the request of the director to increase aid substantially. The museum is a world resource, relied upon not only by its staff but by the scientific community worldwide; clearly, increased support will benefit us all.

J. WILLIAM SCHOPF

*Center for the Study of
Evolution and the Origin of Life,
University of California,
Los Angeles, California 90024, USA*

Destination Mars — a manifesto

Bruce Murray

In proposing a manned mission to Mars, President Bush has allowed Americans to dream again of greatness as a space-faring nation. But dream is more likely to become reality with the help of the Soviet Union.

WHAT happened to the great human adventure in space? After the return of the last Apollo astronauts from the Moon in 1972, the National Aeronautics and Space Administration drifted — no longer was there a compelling destination for Americans in space. The Saturn 5 Moon rocket was decommissioned and its support facilities dismantled, and NASA demanded that the piloted shuttle should be the only access to space for the United States. Planetary exploration was delayed for a decade while President Reagan embraced NASA's simplistic objective of commercial factories in orbit. Finally, in 1986, came Challenger's tragic demise. Americans learned the painful truth that sacrificing astronauts' lives and spending billions of dollars from the public purse requires a nobler purpose than simply driving a space truck to and from low Earth orbit. Undeterred, NASA relentlessly pushed a costly space station as its primary goal. Once again, institutional self-interest propelled NASA to confuse the means with the ends of space endeavours.

And the Soviet Union? After losing the race to the Moon, the Soviets abandoned attempts to send humans any deeper into space. In nearly three decades, they have never ventured much further from the Earth than did Yuri Gagarin. Nor have they developed a new, more powerful launcher for their cosmonauts than the one that carried Gagarin. Instead, the emphasis has been on systematically extending the duration of human stay aboard the orbiting Salyut and Mir space stations. Vladimir Titov and Musa Manarov passed the 365-days-of-weightlessness milestone in the Mir station in 1988.

The space frontier for both countries stagnated a few hundred miles up. Von Braun, Low and Korolov fell ill and died. Dreams of colonies on distant worlds faded. Space bureaucracies on both sides of the Iron Curtain congealed.

Then, on 20 July last year, President Bush announced a new destination for the United States. He urged the renewal of manned ventures beyond Earth orbit, first back to the Moon, and then on to the real goal, "a journey into tomorrow — a journey to another planet — a manned mission

to Mars". Last week, he announced the deadline — 2019, the fiftieth anniversary of the first Apollo landing. Bush is challenging the nation to spend large resources and to risk lives to go to another world. Mars is indeed humanity's best hope for a future beyond Earth; no other planet is potentially habitable, and were Mars as close to Earth as is the Moon,

step out into a gravity field one-third the strength of Earth's). Alternatively, perhaps the entire space habitat will have to be rotated slowly on the journey to produce enough artificial gravity to keep the crew alive and well.

Second, reliable, closed-cycle space habitats are needed. The spaceships would have to be adequate for six-eight

people to live and work in without being resupplied. Unlike the short-duration Apollo flights, much of the Mars crews' air and water must be recycled. The ships will need ten times the 'submerged endurance' of a nuclear submarine (and will not have the opportunity to surface in an emergency), and may even require massive shielding to protect the crews from cosmic rays.

Third, a giant rocket is needed. To travel from Earth's surface to that of Mars requires ten times more propulsion power than the Saturn 5 Moon rocket could deliver. Some¹ even advocate that the United States should develop a nuclear rocket especially for Mars flights, but that would be expensive and difficult, and the main benefit would be to reduce sharply the transit times. Existing rocket technology can do the job otherwise. First, prefabricated sections of the rocket system will have to be ferried up individually and assembled in low Earth orbit. Then many flights by a

rocket cargo transport of 100-tonnes capacity will be needed to carry up fuel for each flight from Earth to Mars.

Since the decommissioning of Saturn 5, the United States has lacked a heavy-lift vehicle. Could the Soviets help? In 1987, they first tested the new Saturn-5-class rocket, the Energia. Like Saturn 5, the Energia flies automatically into space and is not reused. In 1989, it lifted the Soviet shuttle Buran into orbit; pilotless on this occasion, Buran returned safely to Earth entirely under automatic control. This heavy-lift capability and experience of flights of long duration are the strengths of the Soviet Union's space programme. Despite their narrower space technology base, and lack of familiarity with human flight beyond low Earth orbit, the Soviets are further along on some of the technologies for a voyage to Mars. Furthermore, in the mid-1980s Mars became the main objective of the Soviet Union's unmanned

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Mars — the next giant leap for mankind.

territorial rights would already be an issue. But is this a task for the present generation to begin, or one best left for our distant descendants?

In fact, is a journey to Mars even technically feasible? In the 1960s, the United States overcame the unknown to send humans to the Moon and back. We can return if we wish, because the enabling knowledge lingers on long after the ready capability has withered away. But Mars is hundreds of times further away than the Moon. The voyage would probably take some two to three years to complete — fifty times longer than the longest Apollo mission — and to achieve it requires technical advances on three fronts.

First, the harmful effects of prolonged weightlessness must be overcome. A new regimen involving medicine, exercise and special garments might prove adequate to keep crew members healthy for up to a year in space (they must then be able to

USGS/Telegraph Library

planetary exploration programme, a priority that remains in spite of the failure of the attempt in 1988 to land on the Martian moonlet Phobos (a new robotic mission to Mars is planned for launch in 1994). And Mars, not space, is one of the 14 national technical and scientific priorities adopted at the highest level of government in the fall of 1987.

What is not so clear is whether the Soviet space effort will continue to enjoy high priority in the future. Will it survive *perestroika*? Political, ethnic and economic upheavals have slowed, but not stopped, the Energia and Mir programmes. Mr Gorbachev made strong overtures to the United States in 1987 and 1988 for a joint manned spaceflight programme leading eventually to Mars. These overtures were ignored. Indeed, Bush has emphasized US leadership through pre-eminence in space. Speaking on the anniversary of Neil Armstrong's first "small step", he sought to unleash once again the nationalistic energy of the 1960s when John F. Kennedy catalysed American concern over Sputnik and Gagarin. Strong bipartisan congressional support sustained Apollo for a decade. High purpose attracted some of America's best and brightest into NASA to aim for — and reach — the Moon.

In contrast, going to Mars is a two- or three-decade affair. The cost will be an additional 10–15,000 million dollars a year on top of the expense of present civilian space activities. There are many other claimants on the US federal purse, and there is no 'rocket-rattling' Nikita Khrushchev now to challenge the United States into a unified response.

So we now come to the crux of the issue. Given enough time, political will and money, the United States and (probably) the Soviet Union could go to Mars if each so chose. Europe and Japan may want to go along also, but only the two superpowers have the necessary experience of manned flight and the appropriate technology. Yet the heat of national rivalry that powered Gagarin into Earth orbit and Armstrong to the Moon's surface has spent itself — neither country is likely, by itself, to sustain the decades of effort necessary to reach Mars.

The way forward, I think, is a cooperative programme. Substantive collaboration is developing rapidly in robotic Mars exploration for the 1990s, and is winning broadening official support². I believe there are also ways to pool the manned space flight assets of both nations without compromising their separate characteristics. For example, astronauts and cosmonauts could together use the biomedical capability of the United States in long-duration weightlessness studies aboard the Mir space station. That way, the largest technical unknown could be resolved well before the turn of the cen-

tury. If the orbital spaceport for the Mars mission could be reached from both Florida and Baikonur, then added safety, flexibility and economy would result. Energia could ferry the liquid fuel for a joint Mars rocket to be assembled in orbit. That way, US cost projections for going to Mars could be cut by many thousands of millions of dollars; and if the United States were to lead the development of a reliable space habitat, Soviet cost estimates would likewise plummet.

Many people in both countries would regard a truly international attempt on Mars more favourably than a national one. But there are high hurdles, both political and technical, to be surmounted on the way to collaboration. There may be more Lithuanians. And what about technology transfer — won't the United States suffer loss of valuable space technology to the Soviet Union? This objection, for one, was overblown even before the end of the Cold War. As has been pointed out authoritatively³, technology is not so much specific items of hardware that might be part of a joint space system, but rather the techniques for designing and manufacturing key components. Furthermore, the really new technology needed to send humans to Mars — such as the means to enable human beings to spend years in deep space — is of little potential military or commercial value. Robots, not humans, will compete to exploit Earth orbit.

The keys to a robust, enduring partnership are clean technical interfaces and the setting of progressive milestones. If a spaceport developed by the United States proved not to be available to the Soviet Union, then the Soviets would be free to construct their own; and if the United States were to be denied use of Energia, it could develop its own high-capacity transport system. The first trips to Mars would be delayed, of course. But neither country need be a hostage to the other's intentions or stability. Equally important, the first decade need be neither expensive nor risky. For the 1990s, the emphasis should be on joint efforts to acquire the knowledge to make interplanetary flight possible, and to find out more about the environment of Mars. Not until the turn of the century will Mr Bush's and Mr Gorbachev's successors have to make the real commitments for expensive hardware and flight operations.

The initiative for a cooperative flight to Mars is now with the United States. President Bush has the opportunity to carry US foreign policy beyond the Cold War dichotomy of 'allies' and 'adversaries'. Indeed, in March the White House announced it will be discussing the "Human Exploration Initiative" with all space-faring nations, including the Soviet Union. Bush's original nationalistic vision of putting Americans on Mars may actual-

ly find reality as a path-breaking international endeavour.

Both countries have reached an industrial and economic watershed. The long build-up of sophisticated strategic weapons designed for mutual obliteration is ending as the prospect of a Third World War recedes. The aerospace capabilities created to supply the space and terrestrial infrastructure for those weapons may rapidly become legacies of the Cold War. But they remain prized industrial resources — the special expertise of the United States and Soviet Union in rocketry, high-speed flight, human space flight and deep-space exploration still serve to distinguish these nations from Japan and Western Europe.

Continued leadership by the two nuclear superpowers will require them to support non-military aerospace programmes of great technical difficulty and broad popular appeal. Sending humans to Mars would help to retain and enhance some high-technology talent and capability. If real reductions in expenditures for new weapons systems occur, going to Mars will be arguably affordable; the proper currency for that economic debate should be the special skills and facilities necessary for innovative aerospace activities. Will these critical capabilities be retained and enhanced in pursuit of Mars (and kept available for national security in an uncertain future)? Or will they be allowed to disperse or be written off?

The deeper issue for the United States is whether or not manned space flight is to be important in the next century. Manned space flight is intrinsically costly and dangerous. Automated systems can perform practically all functions in space more cheaply and effectively. Remote sensing of Earth, of the Solar System and of the cosmos is robot's work. But high-tech adventure and the experiencing of the unknown for the benefit of Earth-bound humanity are unique attributes of human flight. The proper business of astronauts is exploration.

We can enable a few of our children to walk on a new world — this generation can lay down a challenge to the next by the prospect of travel to Mars, just as Kennedy's legacy of Apollo challenges us today. This time the United States can show the way through international leadership, as well as its own technical excellence. What could be a more promising way to enter the twenty-first century? □

Bruce Murray is in the Division of Geological and Planetary Sciences, California Institute of Technology, Pasadena, California 91125, USA.

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2. *International Cooperation for Mars Exploration and Sample Return* (National Academy Press, 1990).
3. *Balancing the National Interest: National Security Export Controls and Global Economic Competition* (National Academy Press, 1987).

Another gravitational solution found

New solutions of Einstein's equations are rarities, but the latest to appear has the particular interest of making the Big Bang less than a mathematical necessity.

EINSTEIN'S theory of gravitation is generally (and correctly) regarded both as one of the great intellectual achievements of this century and as an awkward starting point for the solution of problems in the real world. The technical difficulty is that Einstein's theory is embodied in a set of non-linear differential equations (strictly, there are ten unknowns, all functions of space and time) which describe allowed patterns of the gravitational field. From any one may be inferred the corresponding distribution of mass or, more generally, energy to which that particular pattern corresponds.

Turning the problem around, so as to tell whether some particular distribution of mass is consistent with a solution of the equations, is difficult for at least two reasons. Because gravitational forces are long-range forces, even the most distant regions of the Universe contribute to local effects. (That is nothing but a reflection of the inverse-square law or of Mach's principle that the most distant galaxies contribute to the gravitational attraction between the Sun and the planets, for example.) Then, because the theory is a relativistic theory which mixes together space and time, not just a distribution of mass but its evolution in time must be specified before one can check whether it is a solution of the equations. One must conceive of a whole cosmology before one can check that it is plausible. It is a hit-or-miss business in which misses are more common than hits.

That is one reason why novel and useful solutions of Einstein's equations are celebrated as if they were new comets or supernovae. That, at least, is the mood engendered by nearly 75 years of frustration in the field.

It was not always thus. Right at the beginning, within a month of learning of Einstein's theory, in 1916, Swartzchild produced a solution of the equations that was not merely mathematically successful but which seemed to describe a reasonable and even realistic kind of Universe, one in which there is a spherically symmetrical distribution of mass. But, until the 1960s, there followed a long period in which more general solutions of Einstein's equations were as plentiful as water in a desert.

For the best part of this century, cosmologists have mostly had to be content with the family of solutions of Einstein's equations produced in 1925 by the Soviet

mathematician Friedman, and which have become the foundation of Friedman–Robertson–Walker (called 'FRW') cosmologies. The underlying assumptions are simple enough. If the properties of space are the same in all directions (isotropic), Robertson and Walker first showed that the distribution of matter would also be isotropic; it fell to Friedman to show what this meant, mathematically, for the pattern of gravitational forces and thus for the distribution of matter and energy.

This is the mathematical basis of most cosmology. (The metric due to R. P. Kerr in 1963 is that on which speculations about isolated black holes are based, and is not strictly the basis for a cosmology.) FRW universes are not static, but are in general either expanding (which provides a natural interpretation of the observed red-shift of radiation from distant galaxies) or contracting. They are also isotropic, which is substantially borne out by observations of the real Universe — at least on sufficiently large scales, radio-galaxies, ordinary galaxies, the X-ray background luminosity and the microwave background radiation appear to be isotropic within reasonable expectations of the measurement techniques. Strictly speaking, FRW universes need not be uniform in the sense that there is no systematic variation of the density of matter from one place to another, but it would then be necessary to suppose that the Earth, or at least the Galaxy, is uniquely placed, at odds with the spirit of the past 300 years.

But FRW universes have a number of special features, some of them mildly embarrassing. For one thing, with reasonable values of the mass density of the present Universe, they do now allow fluctuations of density in the early Universe to condense quickly enough into galaxies of the kind observed quickly enough to explain why the sky we see is filled with them. (That difficulty has been substantially turned by Guth's argument that there was a period of rapid expansion early in the history of the Universe.) A further difficulty is that all FRW universes have a singularity somewhere — if contracting, there comes a stage at which everything is concentrated at a point; if expanding, then they must have begun with something like the Big Bang which has become the contemporary creation legend.

It seems to be an open question whether other kinds of solutions of the equations

might yield radically different but realistic universes. In particular, does the built-in assumption of isotropy force the conclusion that there is a singularity somewhere? A small army of people has by now done its best to explore small variations of FRW space–time, allowing for variations from strict isotropy, but, because these are necessarily approximations, the outcome is also necessarily muddy.

But is there not something to be said more generally? Whatever the difficulties of finding mathematical solutions for Einstein's equations, a feature of the theory of gravitation as distinctive as a Big Bang might be expected to be demonstrable directly, without the cumbersome intervention of algebra. Hawking and, independently, Penrose have argued that there must have been some singularity in the past history of the real Universe. But these arguments seem not to be safe against the discovery of a solution of the equations that lacks a singularity and which is at the same time realistic.

That is the promise of the latest development — the description of a new set of solutions of Einstein's field equations by José M. M. Senovilla from the University of Salamanca, who seems to have found a solution of Einstein's equations which describes a universe evolving from infinitely long ago to infinitely in the future without ever going through a condition recognizable as a Big Bang (*Phys. Rev. Lett.* **64**, 2219; 1990).

Senovilla's universe, which has a kind of cylindrical symmetry, is a remarkably dull place. Time extends from infinity to infinity, with flat and empty space-time at each extreme. But, at times in the distant past, the density of matter and its pressure will increase steadily, until there comes a time when both quantities are a maximum, when the density will decrease again. If this corresponds to the Universe in which we live, the interesting time of maximum density already lies in the past. Whether the new solution is consistent with what is known of the Universe remains to be seen, while something unexpectedly odd may happen on the cylindrical axis of symmetry. But a demonstration that the Big Bang is not a mathematical necessity would be welcome in at least some quarters. So, too, should be Senovilla's proof that there is still a long way to go before the interest of solving Einstein's equations will be exhausted.

John Maddox

The path of drug resistance

Chris Newbold

IN THE early 1960s, the most virulent of the malarial parasites that infects humans, *Plasmodium falciparum*, became resistant to the anti-malaria drug chloroquine simultaneously in both southeast Asia and South America. The spread of resistance from these two foci has since been slow but relentless. The ensuing problems have been enormous — both for residents of endemic areas for whom chloroquine has been used as a first line of treatment, and for medical practitioners in the developed

ation reminiscent of multi-drug resistance (mdr) in cancer cells^{5,6}. The gene responsible for mdr (*mdr*) in cancer cells encodes the MDR protein or P-glycoprotein which, when overexpressed, functions as a transporter to expel drugs from the cells. P-glycoprotein is homologous to a family of ATP-driven transport system proteins originally identified in bacteria, members of which are involved in cystic fibrosis and in pheromone export in yeast^{7,8}. So it was not surprising that the sequence conservation in this family of proteins has been used in two laboratories to identify *P. falciparum* *mdr* homologues^{9,10} — two genes were identified using the polymerase chain reaction and one by oligonucleotide hybridization. These were designated *pfmdr1* and *pfmdr2*. By using malaria *mdr* probes to examine the copy number and transcript levels of *mdr* in chloroquine-resistant and chloroquine-sensitive lines of parasites, it was found that some but not all resistant lines had amplified *pfmdr1* genes and increased *mdr* expression. A link had therefore been established, but cause and effect had not been rigorously demonstrated.

Wellems *et al.* and Foote *et al.* used two different

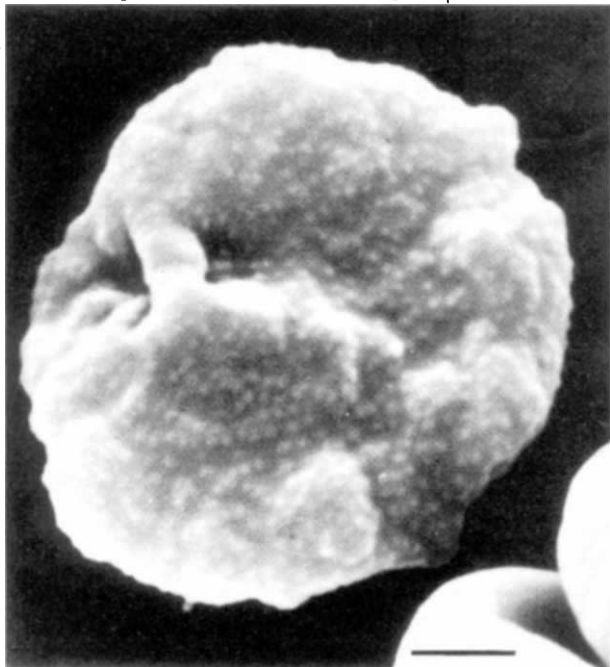
approaches to investigate this relationship further. Wellems *et al.*¹ carried out a single (but very time-consuming) cross between chloroquine-resistant and chloroquine-sensitive clones of *P. falciparum* and assayed the cloned progeny for drug-resistance markers. They looked at the segregation of the *mdr* genes among the progeny by using restriction fragment length polymorphisms specific to one parent and found no relationship between the inheritance of *mdr1* or *mdr2* and the chloroquine-resistant phenotype. The authors therefore conclude that *mdr* is not linked to chloroquine resistance in *P. falciparum*. Foote *et al.*² took a different tack and first identified a substitution 3'

to *mdr1* which was linked to chloroquine resistance, and then identified point mutations which were present in *mdr1* genes of five chloroquine-resistant lines but not in chloroquine-sensitive lines. On this basis the authors obtained several isolates of known chloroquine-resistance status and used the polymerase chain reaction to amplify and then to sequence the areas expected to contain the point mutations. By looking at the sequences they correctly predicted the resistance status of 34 out of 36 isolates — a strong link between *mdr* genotype and chloroquine resistance. Where then was the discrepancy?

Genetic crosses had been previously carried out between rodent malaria parasites rendered resistant to a variety of anti-malaria drugs by *in vivo* drug pressure. In crosses between highly chloroquine-resistant and -sensitive lines, recombinants showed a variety of intermediate levels of drug resistance implying that chloroquine resistance was a result of many mutations at different loci¹¹. In the cross carried out by Wellems *et al.*, the sensitive parent might therefore have had a mutation in *mdr* that was necessary but not sufficient to confer chloroquine resistance. Under these conditions, the *mdr* genotype might be expected not to segregate with the chloroquine-resistant phenotype, because both parents would be 'mdr competent' for resistance. This turned out to be the case, as Foote *et al.* show that the sensitive parent, HB3, has an *mdr* gene of 'resistant'-type sequence. This certainly reconciles some of the contradictory data between the two new studies and is in agreement with previous genetic studies of rodents. It also provides an explanation for why the appearance of chloroquine resistance seems to be rare relative to the appearance of the drug-resistant phenotypes due to point mutations. But it does not completely explain the differences between the new results, for the following reasons.

In the Wellems *et al.* cross, both parents carried different *mdr* mutations that Foote *et al.* would consider competent for

David Ferguson

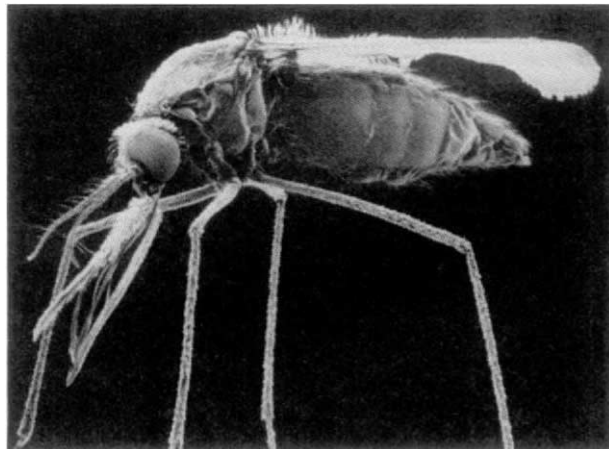


Scanning electron micrograph of a malaria-parasitized red blood cell showing characteristic knob-like structures. Scale bar, 1µm.

world who now find it increasingly difficult to give reliable advice on prophylaxis to travellers. In reports appearing on pages 253 and 255 of this issue, Wellems *et al.*¹ and Foote *et al.*² shed some light on the molecular mechanisms that underlie chloroquine resistance, but come up with contradictory conclusions.

The mode of action of chloroquine as an anti-malarial drug has long been controversial. Although it now seems the main action of the drug is in interfering with the parasite food vacuole³, it has been difficult to ascertain the mechanisms of resistance without being certain of this information. This is in contrast to the situation with pyrimethamine, where resistance often arises as a result of point mutations in the gene for dihydrofolate reductase⁴.

The first clue to a mechanism was that the chloroquine-resistant phenotype is characterized by a greatly increased drug efflux rate which can be partially blocked by calcium-channel antagonists, a situ-



Scanning electron micrograph of a female mosquito, showing the belly swollen after a meal of human blood.

Science Photo Library

conferring chloroquine resistance (Wellems *et al.* were unaware of this at the time). The sensitive parent had a single copy, and the resistant parent four copies. Despite these differences, the phenotypes of the chloroquine-resistant recombinants, whether they possessed a single copy from the sensitive parent or between two and four copies from the resistant parent, were identical with respect to the chloroquine concentration giving 50 per cent inhibition of parasite growth, and the chloroquine efflux rates. So the *mdr* genotype and copy number cannot alone determine these parameters. This suggests that other genes are more important in controlling them, and that the *mdr* mutations may simply facilitate the export of chloroquine, perhaps by altering the affinity of the MDR protein for the drug. Because the rates of accumulation of chloroquine were not measured in these or other related studies, it is quite feasible that other gene(s) affect chloroquine uptake, perhaps by being associated with intracellular chloroquine binding.

The argument for *mdr* being necessary but insufficient for chloroquine resistance is a circumstantial but strong one and relies on the high concordance of mutant alleles and gene amplification with the resistant phenotype. That Foote *et al.* identified two chloroquine-resistant parasites with apparently wild-type *mdr* genes would seem to contradict the basic hypothesis. Unfortunately, these isolates were not clones and it is not clear how many individual products of the polymerase chain reaction the authors sequenced. Also, because they examined only small areas of the genes, they may have missed novel mutations in other regions.

If a multigenic mechanism of resistance is a correct interpretation of the new data, then two further problems arise. First, the high degree of accuracy of phenotype prediction based on *mdr* genotype by Foote *et al.* would be surprising. The authors explain this by the excess of chloroquine-resistant parasites in their sample and the fact that the chloroquine-sensitive lines came from areas where there was no resistance at the time of isolation. Second, Wellems *et al.*'s interpretation of linkage data from a single cross where it was suspected that several genes may be involved is fraught with potential problems.

Both groups nevertheless agree that genes other than *mdr1* are involved in chloroquine resistance. With the cloned

recombinants available from the cross it should now be possible to start to search for these genes by linkage analysis. The biggest stumbling block in these types of studies is the difficulty of carrying out sufficient numbers of genetic crosses with *P. falciparum* to give reliable linkage data. The final answer to whether or not *mdr* in addition to other genes is involved will be arrived at only when a more detailed

OCEANOGRAPHY

Diving into the organic soup

J. R. Toggweiler

ON land, the massive trunks and limbs of trees and woody shrubs make up one of the main repositories of organic matter. In contrast, in the ocean, most plants are microscopic and have no need to produce and maintain massive structures. And so the living biomass of the ocean is tiny when compared with the living biomass on land. Another large repository of terrestrial organic matter is the 'saprophere'¹, the decaying organic matter in soils. The ocean too has a saprophere which consists of dissolved organic matter (DOM) and particulate detritus. The marine saprophere is hundreds of times larger than the pool of living organic material, and roughly the same size as the combined terrestrial organic repositories¹. About two per cent of the marine saprophere consists of large particles that can be examined visually with a microscope. The remaining 98 per cent is contained in dissolved, colloidal and submicrometre particle pools that largely defy characterization. Why there is so much of this material, how it forms and how this huge pool of material relates to the ecology of the ocean are some of the questions that, until now, we have not even begun to answer. On page 242 of this issue, Koike *et al.*², by means of a few simple experiments, have distilled some new facts from this unyielding organic soup.

Koike *et al.* examined oceanic particles with a spherical diameter between 0.38 and 1.0 µm. Most of these particles would be classified as 'dissolved' in routine oceanographic filtrations. The authors determined the number of particles in this size range using a non-destructive particle counter. They counted bacteria in this size range separately using epifluorescence microscopy and were able to show that more than 95 per cent of submicrometre particles are non-living. These particles are very fragile — exposure to high-frequency sound drastically reduces the number of particles that are not bacteria — and flexible enough to sneak through pore sizes nominally only one-quarter of their diameter. Ultra-centrifugation has no effect, indicating that the particles are rather loose aggregations enclosing a

large quantity of water. The highest number of particles in this size range is at the surface; at a depth of 200 m the number of submicrometre particles falls to 1/30 of that found at the surface. Variations with depth show a close association between the number of submicrometre particles and bacterial biomass, chlorophyll and the larger-sized particulate organic carbon caught on glass fibre filters. The measurement techniques do not, however, provide an estimate of the carbon content or mass of material in the submicrometre pool.

Chris Newbold is in the Nuffield Department of Medicine, Institute of Molecular Medicine, University of Oxford, Oxford OX3 9DU, UK

Using filters of ultra-fine pore size (about 0.02 µm) Koike *et al.* found that they could remove 99.5% of these tiny particles from the sea water. Incubations of this highly filtered water inoculated with a small amount of seawater filtered through a 0.6-µm glass-fibre filter produced more bacteria, but no more submicrometre particles. The inoculum in this case included only bacteria and the non-living particles described above; the bacteria in the inoculum multiplied by consuming the dissolved organic matter in the highly filtered solution. But when the inoculum was prepared by passing sea water through a 5-µm filter, 10–20 times more submicrometre particles were produced during the incubation than bacteria. Small animals in the 5-µm filtrate apparently began eating bacteria and presumably produced the submicrometre particles by egestion or in the process of lysing bacterial membranes.

This finding is consistent with the arguments of Jumars *et al.*³ regarding the origin of the organic matter fuelling the heterotrophic microbial community in the sea. Between 20 and 40 per cent of the primary production of oceanic plants passes through the bacteria⁴, and it is not clear how this happens. Many have argued that the organic substrates feeding the microbial community come directly from the phytoplankton as dissolved organic exudates⁵. According to this view, these relatively simple compounds, if not immediately utilized by bacteria, can react to form complex unreactive geopolymers, which build up in the ocean and account

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for the huge size of the oceanic DOM pool. In contrast, Jumars *et al.* argue that marine zooplankton, in the process of eating phytoplankton cells, other animals or bacteria, egest or leak unassimilated materials in sufficient quantities to account for the level of microbial activity observed in the ocean. It is not clear, however, that dissolved organic material associated with animal egesta will persist long enough in the ocean to accumulate as high concentrations of DOM. Despite these shortcomings, the animal model is attractive because it easily accounts for the relatively low C/N ratio in the oceanic DOM, and does not require any hypothetical chemical reactions.

Not too many years ago the marine saposphere was thought to consist mainly of very refractory organic compounds which circulated through the oceans for thousands of years before breaking down⁷. The dissolved organic pool in the ocean was thought to be equivalent in many ways to the pool of decaying organic matter in soils. Within the past few years, Suzuki *et al.*⁷ and Sugimura and Suzuki⁸ have perfected a technique for the catalytic oxidation of marine organic matter and as a result of this work, ocean chemists have come to recognize a new pool of organic material. The new pool rivals the old pool in its size (such that the total pool is now twice the size of the old pool), but not in its inactivity. Whereas the old pool of DOM was more or less evenly distributed throughout the ocean, the new pool is concentrated near the surface and appears to be actively involved in the biogeochemical cycling of carbon and nitrogen⁹.

One largely unrecognized aspect of Suzuki and Sugimura's work is their attempt to break the DOM pool down by size fraction using gel filtration. The figure shows a water-column profile of total dis-

solved organic carbon (DOC) from the western Pacific broken down into six size fractions, where size fraction 6 is the largest. Most of the DOM extracted by older methods is composed of smaller molecules, corresponding roughly to size fractions 1 and 2. Most of the gradient in DOC between the upper ocean and the deep ocean is contained in the larger size fractions 3–6. To first order, the larger sizes represent the difference between the old and new material.

Fractions 5 and 6 largely disappear in the upper few hundred metres of the ocean, as do Koike *et al.*'s submicrometre particles. The largest molecular weight fraction (>100,000 daltons) probably overlaps the size range inhabited by the Koike *et al.* particles, such that size 6 and the submicrometre particles may indeed be the same material. Break-up of fragile submicrometre particles into smaller and more typically 'dissolved' entities could conceivably account for the larger pool of smaller compounds that comprise the bulk

GEOCHEMISTRY

Granulites and the lead paradox

Robert Zartman

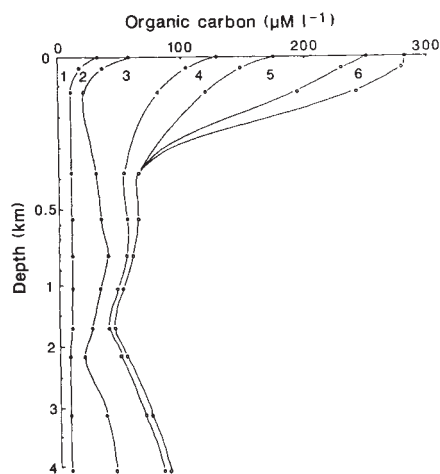
NEW isotopic and chemical data for rocks that are rarely seen at the surface of the Earth, but which may play an important role in understanding the evolution of the continental crust, are always exciting. And Rudnick and Goldstein¹ now report on the lead isotopic composition of three suites of mafic granulite xenoliths — essentially doubling our knowledge of lead isotopes in this largely neglected rock type originating in the lowermost crust.

Petrologically, most mafic granulites appear to be variants of metamorphosed basaltic rocks, that presumably have been injected into the crust from the underlying mantle. Geobarometry and geothermometry suggest that the granulites crystallized at depths of 35–50 km and temperatures substantially above the normal continental geothermal gradient. Together with Sr and Nd isotope ratios and other chemical data on the same xenoliths, these new analyses challenge previous ideas about the composition, age and mode of formation of the Earth's continental crust. Although the xenolith suites occur in areas with Proterozoic basement rocks (about 1,500 million years old), the isotopic evidence points to a much younger emplacement age for the precursors of the mafic granulites. If mafic granulites constitute a major lithostratigraphic unit in the lower crust, it may be necessary to contemplate a crust that is more mafic in composition and younger in age than usually assumed, and one that can be progressively thickened from below by the intro-

duction of mantle-derived magmas. of Suzuki and Sugimura's DOM pool (size fractions 3, 4 and 5). To the extent that this might be true, the experiments of Koike *et al.* may herald the start of a new approach for the study of the marine saposphere. □

J. R. Toggweiler is in the Geophysical Fluid Dynamics Laboratory, Princeton University, PO Box 308, Princeton, New Jersey 08542, USA.

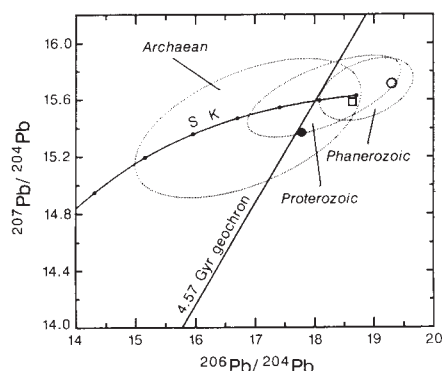
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Concentration of total dissolved organic carbon with depth at 5° N 135° E in the western Pacific divided among six size classes: 1, <1,800; 2, 1.8–4 × 10³; 3, 4–20 × 10³; 4, 2–6 × 10⁴; 5, 6–10 × 10⁴; 6, >1 × 10⁵ daltons (from ref. 8).

duction of mantle-derived magmas.

Much of the earlier modelling of the lower crust, including my own², has been based on the premise that felsic granulites — often of great age and very low μ (²³⁸U/²⁰⁴Pb ratio) — are the dominant rock type below mid-crustal levels. (The low value of μ will retard the further growth of radiogenic lead.) A perplexing problem of Pb isotope systematics was thus avoided. The difficulty comes from the so-called lead paradox³, which arises when the apparent bulk-Earth Pb isotopic composition plots off the geochron (the line on ^{a207}Pb/²⁰⁴Pb versus ²⁰⁶Pb/²⁰⁴Pb diagram representing the closed-system lead isotopic evolution of the Earth since its origin 4.57 thousand million years ago, see figure). Because the fields of the upper crustal and mantle reservoirs are fairly well-defined and lie to the right of the geochron, for balance, a complementary reservoir to the left of the geochron is needed. Old, low- μ lower crust composed mainly of felsic granulites held promise of offering a solution to this paradox⁴. By contrast, from Rudnick and Goldstein's results it seems that the lower crust has a younger 'tectonothermal' age and an isotopically less-retarded lead content than has usually been assumed. If their findings are generally applicable, we must almost certainly look elsewhere for the missing complementary reservoir (possible candidates are the subcontinental lithosphere, lower mantle or core) — or, alternatively, entertain the notion of a significantly younger age for



The Pb isotopic fields of young (less than 200 million years old) felsic plutonic and volcanic rocks derived from lower continental crust of Archaean ($n = 122$, $\alpha = 17.12$, $\beta = 15.32$), Proterozoic ($n = 243$, $\alpha = 17.89$, $\beta = 15.52$) and Phanerozoic ($n = 347$, $\alpha = 18.76$, $\beta = 15.65$) age — n , number of samples; α , average $^{206}\text{Pb}/^{204}\text{Pb}$; and β , average $^{207}\text{Pb}/^{204}\text{Pb}$. Also shown is the Pb isotopic composition of the lower crust (solid circle), upper crust (open circle), and total crust (square) as modelled by Zartman and Haines⁵. The preferred values of Rudnick and Goldstein¹ for the corresponding reservoirs lie slightly to the left of these points. The 4.57 thousand million year (Gyr) geochron and Stacey and Kramers⁶ growth curve (labelled S–K, ticks at 0.4-Gyr intervals) are shown for reference.

the Earth as a whole.

Another line of evidence relating to the Pb isotopic character of the lower crust seems increasingly relevant, and also suggests a total crust displaced well to the right of the geochron in Pb isotope space. Because of the rarity of actual samples of the lower crust — chiefly limited to kimberlite- and basalt-hosted xenoliths and a few tectonically uplifted terranes — considerable interest has focused on the Pb isotope ratios of young felsic plutonic and volcanic rocks derived from lower crustal sources of various ages and tectonic settings (see figure). This worldwide data set, which includes hundreds of analyses, should minimize sampling bias, and, consequently, can be used to calculate an average Pb isotopic composition for large volumes of the lower crust. Despite some uncertainty in age and Pb concentration of the source rock, the evidence so obtained for a time-integrated μ -value in the lower crust of 6–8, rather than about 1 or less, is overwhelming, that is the lower crust itself lies close to the geochron. Although continuing to support a somewhat diminished isotopic evolution compared to the upper crust, the highly unradiogenic reservoir required to solve the Pb paradox has not been identified in the young igneous rocks. Still, very-low- μ felsic granulites do occur fairly commonly as xenoliths and in some deeply exposed terranes, and they cannot be ignored as a component of continental crust.

The answer to this apparent contradiction in the composition of the crust may lie with the mafic granulites themselves.

Plants in the post

EARLIER this month the United Nations Postal Administration issued a set of six stamps commemorating the work of its sister body UNIDO with medicinal plants. Over the past five years, UNIDO (United Nations Industrial Development Organization) has been involved in promoting the industrial production of medicine from wild plants in developing countries as a low-cost, readily available alternative to the more

expensive conventional medicines marketed in developed countries.

The stamps, illustrated by watercolours from *Botanic Magazine* established by the eighteenth-century botanist William Curtis, each show a plant noted for the beneficial effect of its products on health. Depicted here is *Cinchona officinalis*, an evergreen tree, native to the Andes of Ecuador and Peru. It is widely cultivated for the dried bark of its stems and roots, known as cinchona or Peruvian bark, the source of quinine and other anti-malarial alkaloids. The tree was named after the Countess of Chinchon, wife of the Viceroy of Peru, who is said to have been cured of tertian fever in 1638 by a preparation of the bark.

Quinine has been largely replaced by syn-



thetic anti-malarial drugs developed in response to the flow of quinine from Java being cut off by the Japanese during the Second World War; resistance to these drugs is now a large problem (see pages 202, 253 and 255).

The UN Postal Administration is the only body in the world without sovereign territory that has the right to issue its own stamps. The new issue is available in three currencies, but can be used for postage only from certain UN headquarters — unfortunately, not most people's usual stamping ground.

Peter Tallack

Bohlen and Mezger⁷ have summarized the argument for a three-tiered crust in which, in addition to the unmetamorphosed to moderately metamorphosed upper crust, high-grade felsic granulites comprise the middle crust, and under-plated mafic granulites comprise the lower crust. If the mafic granulites do represent a later addition to the crust and acquire their isotopic signatures from invaded older rock without an abnormally low μ value, a reconciliation between the measurements of mafic granulites and the indirect evidence provided by young igneous rocks becomes possible. Preserved enclaves of ancient, low- μ felsic granulites may be less abundant than once thought, and may also be mainly restricted to intermediate crustal levels.

As Rudnick and Goldstein point out, any attempt to extrapolate their limited data to an average crustal Pb isotopic composition leaves many questions unanswered about the structure, compo-

sition and age of a crust, which, apparently, grows both laterally and vertically. A significant 'younging' of the continental crust with depth will require a re-evaluation of most models that assume that the age of the crust does not change with depth. In the process of redefining the nature of the deeper continental crust, we appear to find ourselves back with the Pb paradox and with renewed speculation on its cause. □

Robert Zartman is in the US Geological Survey, Branch of Isotope Geology, Denver, Colorado 80225, USA.

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Dynein and the kinetochore

Richard Vallee

THE kinetochore — the site at which microtubules attach to chromosomes during mitosis — is crucial for normal chromosome segregation; upon its performance hangs the fate of the progeny of cell division. It has been shown by electron microscopy to be a trilaminar plate situated at the primary constriction site of the condensed eukaryotic chromosome. It is coincident with the centromere, the genetically defined centre of meiotic recombination. But just what does the kinetochore consist of and what does it do? Is it simply a passive attachment site for microtubules, or does it play an active role in mitosis? New insight into the behaviour of chromosomes in the early stages of cell division, reported by Rieder, Alexander and Rupp¹, has revealed the relationship between kinetochore and microtubule to be highly dynamic. And now, as described on pages 263 and 266 of this issue by Pfarr *et al.*² and Steuer *et al.*³, the force-producing ATPase cytoplasmic dynein has been localized to the kinetochore.

Chromosomes are involved in a series of directed movements during mitosis (Fig. 1). In prometaphase, chromosomes attach to spindle microtubules and, at least in some clear cases, can be seen to

gather at the spindle poles¹. Subsequently, through a slow bidirectional shuffle, they migrate towards the mid-zone of the spindle and form the metaphase plate. Finally, the true business of mitosis takes place. In a much more coordinated and stately manner the chromosomes proceed towards the spindle poles during anaphase. The first part (anaphase A) is thought to involve the specific shortening of kinetochore-associated microtubules⁴, while the second (anaphase B) involves elongation of the entire mitotic spindle⁵.

What is the role of the kinetochore in all of this? One school of thought has it that it is a passive structure with the remarkable ability, nonetheless, to hold on to microtubule ends while the microtubules shorten⁶. This view is based on the behaviour of microtubules attached to isolated chromosomes *in vitro*. The microtubules were induced to shrink while maintaining their attachment to the kinetochore. The approach of the microtubules to the kinetochores mimicked *in rate* (1–12 $\mu\text{m min}^{-1}$) and direction the relative movement of chromosomes and microtubules during anaphase A. Importantly, this *in vitro* behaviour occurred in the absence of an energy source such as ATP, suggesting that microtubule disassembly was sufficient to drive chromosome movement during anaphase A. A role for force-producing 'motor molecules', at least in the movement observed *in vitro*, would seem to be ruled out.

In similar experiments using stabilized microtubules, however, ATP-dependent chromosome movement was observed, as indicated by a shift in the attachment site of chromosomes from one end of the microtubule to the other (from minus end to plus end)⁷. This movement has now been detected in real time at rates of some 2–3 $\mu\text{m min}^{-1}$ (ref. 8). The direction of movement was opposite to that seen in anaphase A, but might in part explain congression of chromosomes to the metaphase plate during prometaphase. Because stabilized microtubules were used in these experiments, their behaviour could not have been driven by microtubule assembly or disassembly; rather, the requirement for ATP is more readily explained by a kinetochore-associated motor molecule.

Rieder *et al.*'s recent work¹ provides

dramatic evidence for rapid chromosome movement along microtubules *in vivo*, and strongly implies that motor molecules have a role in this process. During prometaphase in newt lung cells, occasional laggards were observed among the chromosomes as they gathered at the spindle poles. Because of their spatial and temporal segregation from the rest of the chromosomes, the behaviour of the

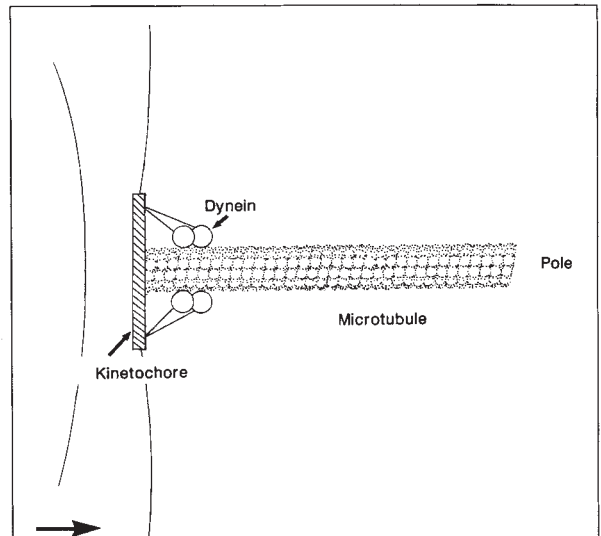


FIG. 2 Possible arrangement of dynein in the kinetochore. Two-headed dynein molecules are shown tugging on the end of a microtubule, whose disassembly could govern rate of movement. (From ref. 14.)

lagging chromosomes could be monitored more clearly. Abruptly, these chromosomes attached to individual microtubules, and then migrated towards the spindle poles at rates up to 55 $\mu\text{m min}^{-1}$, a rate much faster than the maximum rate observed for microtubule depolymerization. Serial section electron microscopy revealed that the microtubules either passed through (or, more probably, across) the face of the kinetochore. This suggests that the kinetochore runs along the microtubule, similar to the behaviour of membranous organelles engaged in rapid axonal transport. The comparable rates of chromosome and organelle movement further imply a common mechanism involving organelle- or kinetochore-associated 'motor molecules' rather than microtubule disassembly. The obvious candidate for such a motor molecule is cytoplasmic dynein, which produces force in the appropriate direction to move chromosomes towards the spindle poles⁹.

Now, Pfarr *et al.*² and Steuer *et al.*³ have obtained more direct evidence that cytoplasmic dynein is, indeed, associated with the kinetochore as well as with other spindle structures. Both groups used an essentially similar approach, preparing antibodies to two different subunits of this multisubunit enzyme — the heavy chains of relative molecular mass (M_r) 400,000 and an intermediate chain of M_r 70–79,000 (ref. 10) — and using them

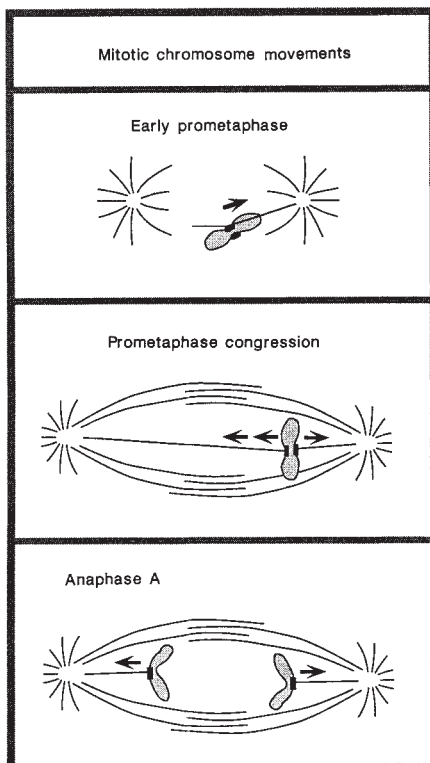


FIG. 1 In prometaphase, the chromatid pair is first drawn to the spindle pole¹, then migrates towards the centre of the spindle. At the onset of anaphase, the chromatids separate and migrate slowly toward the spindle poles.

for immunofluorescence microscopy of dividing mammalian cultured cells, HeLa and PtK cells by Pfarr *et al.*² and chicken embryo fibroblasts by Steuer *et al.*³. Staining of kinetochores was strikingly apparent in prophase and persisted through prometaphase. Staining of the forming spindle poles was also seen in prophase, and spread outwards along the polar microtubules through prometaphase. By anaphase, kinetochore staining was less clear, possibly obscured by the increasing microtubule staining, though perhaps reflecting the disappearance of dynein from the kinetochore.

What are the mechanistic implications of these results? First, the relatively strong staining of the kinetochore during prometaphase supports the idea that dynein has a role in the rapid chromosome-to-pole movement observed by Rieder *et al.*, and may explain this phenomenon.

Could kinetochore dynein explain other chromosome movements as well? The bidirectional oscillations seen during prometaphase chromosome congression are reminiscent of the bidirectional movements of membranous organelles in cells. A report of a cytoplasmic dynein in the giant amoeba *Reticulomyxa*, capable of bidirectional force-production, has appeared¹¹, though mammalian dyneins have, so far, been found to produce force only in the 'retrograde' direction.

Anaphase A movement of the chromosome towards the spindle pole fits the direction of force production for dynein, but is far slower (about $1 \mu\text{m min}^{-1}$) than dynein-mediated movements ($1\text{--}2 \mu\text{m s}^{-1}$). This could in part be explained by the abutment of the kinetochore with the end of the microtubule during anaphase,

which could serve to retard chromosome movement (Fig. 2).

Dynein joins a small class of proteins that have been identified in the kinetochore by biochemical and immunological means^{12,13}. Its finding adds new excitement to the kinetochore story, implying that many of the complex events during mitosis may be understood through the behaviour of a limited number of molecules, whose properties in some cases may already be known. The job now will be to define just how and when dynein may act during mitosis, and how its behaviour may be integrated with that of other potential mitotic motors (see 3 May News and Views¹⁵). □

Richard Vallee is in the Cell Biology Group, The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545, USA.

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COSMOCHEMISTRY

Graphite in meteorites

J. Nuth

As described on page 238 of this issue¹, Amari, Anders, Virag and Zinner have recovered circumstellar graphite grains from a primitive meteorite. This remarkable achievement is the third in a series of chemical separations by Anders and colleagues, who have previously recovered circumstellar diamonds² and silicon carbide³ from similar meteorites. Although graphite was once considered to be ubiquitous in the interstellar medium and to account for more than 80 per cent of the cosmic carbon abundance⁴, less than one part in 10,000 of the total carbon in the meteorite is graphite — silicon carbide is three times more abundant, and diamond 200 times more abundant.

In addition, Amari *et al.*¹ have placed an upper limit on the possible abundance of amorphous carbon in the meteorite which, depending on the relative reactivity

of amorphous carbon, is between 5 and 50 times higher than the abundance of graphite. Barring some unknown process in either the interstellar medium or the primitive solar nebula that would selectively destroy one or more of the aforementioned forms of carbon (that is, diamond, silicon carbide and graphite), these results imply that organic carbonaceous grains may account for most of the carbon abundance in the interstellar medium.

Amari *et al.* were searching for the carrier of neon-E(L), nearly monoisotopic ²²Ne (resulting from the decay of ²²Na), released at relatively low temperature from carbonaceous meteorites, and used this noble gas component as a tracer to guide their chemical separations. As ²²Na has a half-life of only 2.6 years and as it may be formed in the ejecta of novae and

red giants, it must have been incorporated into carrier grains quite soon after the nucleosynthesis event in which it was produced in order to have preserved its monoisotopic identity. Once released from its carrier, neon-E would quickly be diluted by other naturally occurring neon isotopes and would be impossible to recover in pure form.

From the wide range of ¹²C/¹³C and ¹⁴N/¹⁵N ratios observed in individual grains, Amari *et al.* have shown that the graphite recovered must have formed in a relatively large number of separate, carbon-rich environments. The presence of neon-E(L) in such grains implies that they were never subjected to a harsh environment (for example, temperatures in excess of 600 °C) for any appreciable time, from their formation in circumstellar outflows to their recovery in the laboratory. These results may therefore place an upper limit on the temperature of the solar nebula in the region of meteorite formation. They also call into question the applicability of recent calculations of the destruction efficiency of supernova shocks on the average interstellar-grain population⁵. According to such calculations, grains are destroyed about ten times faster in the interstellar medium than the rate at which they form in circumstellar outflows. This would mean that only five circumstellar grains in 100,000 would survive residence in the interstellar medium. Graphite, the least abundant circumstellar component yet isolated from primitive meteorites, is itself twice as abundant as would be predicted on this basis.

Ion-probe measurements of individual graphite grains larger than about 100 nm in diameter have revealed a wide range of ¹²C/¹³C ratios, from 1,440 to as low as 8. These values can be compared with the average value of this ratio for objects in the Solar System (89) and with the highest ratios previously observed in carbon stars (97) or meteorites (159). Very high values of ¹²C/¹³C may form in stars that are very deficient in metals: no such star has ever been observed. The graphite grains isolated by Amari *et al.* must have formed more than five thousand million years ago, possibly in the death throes of a carbon-rich red giant. These grains may therefore reflect the chemical and isotopic composition of matter in our Galaxy over the past 5–10 thousand million years. The presence of a number of grains with high ¹²C/¹³C ratio may be related to galactic chemical evolution and the general increase in the metal content of younger stars. Systematic study of the composition of such grains may provide valuable information on the rate of such an increase.

Anders has done a remarkable job using noble gas tracers to guide him and his co-workers in separating specific carrier phases from meteorites; interest-

ingly, each of these phases formed in a carbon-rich environment. Are there reasons to suspect that circumstellar silicates cannot survive passage through the interstellar medium but that graphite, silicon carbide and diamond can? Is it possible that no identifiable noble gas component is formed in oxygen-rich environments or that silicate grains cannot bind such components? Perhaps noble gases are not the proper tracer to use in isolating presolar oxide grains. Unfortunately, it may never be possible to use chemical means to separate presolar oxide grains from silicates formed in the solar nebula because there is no reason to suspect that these two grain populations would react differently. Future studies of presolar grain components in primitive meteorites may rely on direct *in situ* detection of the component and any associated grains in the meteorite matrix. Isolation and characterization of presolar SiC grains by Anders² and colleagues have made it possible for Robert Walker and co-workers at Washington University to develop a strategy to conduct one such preliminary *in situ* survey. Walker *et al.*⁶, who so far have examined only 0.169 cm² of the matrix of the Cold Bokkeveld meteorite, have discovered 16 SiC grains ranging in size from 600 to 2,400 nm in diameter. It is possible that the characteristics of presolar diamond and graphite grains may eventually suggest methods for the *in situ* detection of these components in a meteorite matrix as well, and that such studies might possibly reveal associated interstellar silicates.

Anders and his collaborators have opened the door to an entirely new field of astrophysics. Laboratory study of presolar grains has the potential to revolutionize our understanding of nucleosynthesis processes in individual stars, galactic chemical evolution and grain survival in the interstellar medium, to mention only three examples. The potential impact on many other areas of astrophysics is still unknown. Few astrophysicists expected that, in their lifetimes, interstellar materials would be analysed in the laboratory, and as yet there are few quantitative predictions of what will be revealed by such analyses. The fun has just begun. □

J. Nuth is in the Laboratory for Extraterrestrial Physics, NASA/Goddard Space Flight Center, Code 691, Greenbelt, Maryland 20771, USA.

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Naked stars and hot meteorites

Paul Weissman and John Wasson

TRYING to understand the formation of stars and solar systems is a frustrating business. Our own Solar System is 4,500 million years old, and most of the evidence relating to its origin has been obliterated by a variety of planetary processes (impacts, chemical differentiation and tidal heating, to name but a few). On the other hand, the best current examples of solar-type star formation are so far away, about 150 parsecs, that they are not resolvable with Earth-based telescopes at scales less than twice the diameter of our

per year. But Strom reported that a survey of T Tauri stars showed a diminishing of infrared excess, interpreted as a decrease in protoplanetary disk activity, for stars further along their Hayashi evolutionary tracks (that is, for older protostars). After about ten million years, the nebula disks appear to be largely gone. This new class of object was discovered during a survey at X-ray and ultraviolet wavelengths by Fred Walter (State University of New York, Stony Brook). He named them “naked T Tauri stars” (although one

IMAGE
UNAVAILABLE
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REASONS

Triton, Neptune's largest moon, as seen by Voyager 2. The large south polar cap can be seen at the bottom. The darker colour north of the cap is thought to be an effect of ultraviolet light and magnetospheric radiation on methane in the atmosphere and on the surface.

own planetary system. It's enough to make a scientist switch to studying cold fusion. Nevertheless, there are still those who try. And the third incarnation of the Protostars and Planets* meeting in Tucson, Arizona (a gathering of astronomers and planetary scientists that takes place every six years), showed that there was still good reason to hope.

One of the most interesting advances since the last meeting is the observational determination of a limit on the timescale over which the primordial solar nebula dispersed. Stephen Strom (University of Massachusetts) described observations of T Tauri stars, the archetypal solar mass, pre-main-sequence stars. T Tauri stars are characterized by large infrared excesses from extended disks of orbiting protoplanetary material, excess ultraviolet radiation and strong stellar winds with mass-loss rates of 10^{-8} to 10^{-5} solar masses

per year. But Strom reported that a survey of T Tauri stars showed a diminishing of infrared excess, interpreted as a decrease in protoplanetary disk activity, for stars further along their Hayashi evolutionary tracks (that is, for older protostars). After about ten million years, the nebula disks appear to be largely gone. This new class of object was discovered during a survey at X-ray and ultraviolet wavelengths by Fred Walter (State University of New York, Stony Brook). He named them “naked T Tauri stars” (although one

prominent astronomer said that she was “too much of a lady to ever use that term”, and preferred “weak-line T Tauri stars”). The absence of detectable disks in the infrared does not mean that the dust has somehow been blown away, but rather that it has been accreted into larger orbiting particles, thereby decreasing its effective surface area below that detectable with current ground-based instruments. And the new particles do not have to be very large: if the dust was initially all one micrometre in radius, accretion into 10-micrometre particles would be sufficient to make it undetectable. But the suggestion, of course, is that once accretion starts, it will continue, eventually resulting in the growth of planetesimals, and then planetary bodies.

The ten-million-year limit becomes even more important when one considers what it might mean for the accretion of Jupiter and Saturn. Like the Sun, these two planets consist primarily of hydrogen

* *Protostars and Planets III*, 5–9 March 1990, Tucson, Arizona.

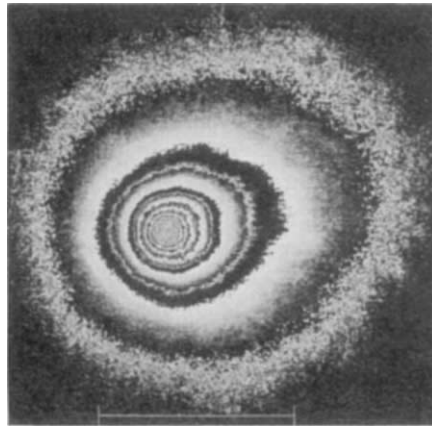
and helium. They must have formed very early, before the gas in the nebula had time to disperse. In addition, it seems that Jupiter had to form ahead of the terrestrial planets to forestall the growth of a large planet in the asteroid belt, and to stunt the growth of Mars. The naked T Tauri stars appear to confirm the timescale for this general scheme.

Investigations along similar lines were reported by Suzan Edwards (Smith College) who noted that as the dust disks around the young stars faded from view, the intensity of mass outflows also decreased. This suggests that the energy source for the strong stellar winds is the accretion of disk material onto the star. When the disk material begins to accrete into planetesimals, the infall stops and the resulting mass outflows also halt.

Another sign of hope in Tucson was the possible resolution of a long-standing problem between protosolar nebula modellers and meteoriticists. As discussed at the meeting by Herbert Palme (Max Planck Institute, Mainz), the chondritic meteorites were subject to very high nebular temperatures during their formation. The chemical fractionations in all chondrites seem to require temperatures above 1,000 K, and some rare-earth element fractionations indicate values exceeding 1,500 K. In contrast, numerical simulations of star formation rarely yield nebula temperatures as high as 500 K at a typical asteroidal distance of 2.5 astronomical units (AU) from the forming protostar.

Thus, the meteoriticists were pleased to learn from Lee Hartmann (Harvard-Smithsonian Center for Astrophysics) of recent evidence regarding FU Orionis-type stars. FU Orionis objects undergo outbursts during which their luminosities increase by factors of hundreds or thousands, leading to temperatures greater than 1,500 K throughout the inner 3 AU of the stellar system. There was general agreement among the astrophysicists at Tucson that the eight recognized FU Orionis objects are all bona fide T Tauri stars, but no agreement on the mechanism responsible for the prodigious outbursts; one suggested mechanism is a sudden enhancement of the protoplanetary disk accretion onto the growing protostar. It was also agreed that our Sun had experienced a T Tauri phase, but whether it went through FU Orionis-type outbursts is still an open question. It is not certain that meteorites could have formed, and their parent bodies been preserved, if these highly luminous periods were powered by turbulent, disk-type accretion.

But if new data helped alleviate old problems, they also raised new questions. Since the last meeting, Voyager 2 has provided insights into the nature of the outer planets and their satellite systems. The Vega and Giotto explorations of comet Halley yielded fascinating compositional



Ultraviolet image of comet Halley showing the huge hydrogen cloud surrounding the nucleus. Scale bar, 10 million kilometres.

evidence on cometary nuclei, believed to be representative of the primitive materials in the primordial nebula. But whereas Triton and Pluto, among our Solar System's most distant large bodies, have large quantities of methane on their surfaces, comet Halley had little or possibly even no methane. How did one class of body come to have carbon in the reduced state, whereas the other has carbon only in the oxidized state, if they formed in the same area of the nebula? Perhaps planetary processes have reprocessed carbon monoxide into methane at Triton and Pluto. But there are no firm indications as to what those processes may be. Or perhaps the comets formed somewhere else, further out in the nebula in the proposed Kuiper Belt of comets beyond Neptune, or even in distant nebula fragments orbiting at Oort-cloud distances of $5-50 \times 10^3$ AU.

A tantalizing piece of evidence which ORNITHOLOGY

Changing migration behaviour

Jeremy J. D. Greenwood

WHAT controls migratory behaviour in birds? Is migration something so deeply embedded in inheritance that the pathways followed today largely reflect colonization routes as bird populations followed the retreating glaciers northwards? Or is it flexible, with the behaviour of individual birds being determined largely by environmental factors? The field evidence is inconclusive, so experiments are essential in order to tackle these questions. Peter Berthold and his colleagues at the Vogelwarte Radolfzell in Germany have been conducting an experimental programme for many years, and have now produced results that show, for one population of one species at least, that migratory behaviour is under strong genetic control but that there is considerable genetic variation within the population¹.

The experiments depend on the consid-

erable aptitude the Radolfzell team has for maintaining breeding populations of small insectivorous birds in captivity and on the careful choice of study species^{2,3}. They involve the study of migratory behaviour in caged birds by observing 'migratory restlessness' — the hopping and fluttering movements displayed by caged birds at the times of year, and times of day or night, at which they would normally migrate. The behaviour lasts for the same length of time that normal migration would last and is orientated in the correct direction, even to the extent of altering direction at the appropriate time in those species that change direction during the course of migration^{2,3}.

Berthold and his colleagues have previously shown that crosses between fully migratory German blackcaps (*Sylvia atricapilla*), and those from partially migratory African populations or from the

As the faithful departed from Tucson, they surely must have been in an upbeat mood. Although some researchers had given talks as opaque as the interstellar clouds they were discussing, most had made a genuine effort to achieve real communication between the astrophysics and planetary sciences communities.

The next meeting is certain to be strongly influenced by results from the Hubble Space Telescope, and the soon-to-be-launched Infrared Space Observatory. What new insights will the orbiting observatories bring? One can only wait with eager anticipation for that particular curtain to rise. □

Paul Weissman is in the Earth and Space Sciences Division, the Jet Propulsion Laboratory, Pasadena, California 91109, USA. John Wasson is in the Institute of Geophysics and Planetary Physics, the University of California, Los Angeles, California 90024, USA.

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Swallows gathering — how much is migratory behaviour determined by inheritance?

non-migratory Cape Verdean population, produce offspring in which the extent and pattern of migratory restlessness is intermediate between that of the parental populations^{2,3}. The differences between the populations are thus largely genetic.

But what of variation within populations? In some, sex and age may largely determine whether an individual migrates but there are also many cases where there is a dichotomy within sex and age classes, with some individuals migrating and others not. Various studies have suggested that the dichotomy is at least partly genetic in some species^{4,5} and the new experiment has dramatically confirmed that this is indeed the case. The Radolfzell workers used blackcaps from a French population in which about 75 per cent of the birds migrate. They applied selection, breeding in one line only from birds showing migratory restlessness and in another only from those that did not. In the first line, 100 per cent of the population showed migratory restlessness after only three generations; in the other, migratory restlessness was eliminated in six generations.

The heritability of migratory behaviour revealed in this experiment is 0.6–1.0, among the highest values recorded for quantitative characters in bird populations⁷. The ecological conditions experienced during the winter by migrants and residents are very different and the hazards of migration are great, meaning that migration behaviour is probably under strong natural selection. Yet, according to empirical evidence and standard quantitative genetics theory, the heritability of characters strongly associated with fitness is low, because selection erodes the additive genetic variance that gives rise to heritability. The paradox is perhaps explained by the type of selection operating in partially migrant populations. The coexistence of migrants and non-migrants is probably the result of the

over-winter survival of at least one of the subpopulations being density dependent; in consequence, the relative fitness of each type declines as it becomes more frequent, leading to a stable equilibrium at the point at which fitnesses are the same. But any change in selection, such as that in the experiments, results in a rapid response because of the persistent genetic variation.

The rapidity of the response fits in with observations that the numbers of migrants in Belgian populations of stonechats (*Saxicola torquata*) are higher after hard winters, during which mortality of residents may be assumed to be high⁸. As Berthold and his colleagues suggest, it also has implications for the effects of global warming on bird populations: the migrations of some species may rapidly evolve as their environment changes, altering the ecological balance between them and their competitors. Given the extra problems that European migrants may face because of the increasing aridity of the Mediterranean region, through which they must pass to reach Africa⁹, we can hope that the Radolfzell group will not only discover more about the genetic architecture of migration behaviour in blackcaps, but extend their work to other species. □

Jeremy J. D. Greenwood is at the British Trust for Ornithology, Beech Grove, Station Road, Tring, Hertfordshire HP23 5NR, UK.

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Deep liquid gold

ALONG the central ridges of the big oceans, where the tectonic plates are separating, intriguing aqueous volcanoes called 'black smokers' occur. Sea-water percolates through the cracking and extending rock, down to the hot magma beneath. At such high temperatures and hydrostatic pressures water becomes a supercritical fluid, and a most ferocious solvent. It dissolves sulphides and other minerals from the magma, convects up the exit pipe of the black smoker, and erupts from the sea-bed as a superheated solution. As it cools and mixes back into the cold ocean, its dissolved and suspended minerals precipitate as black 'smoke'. Impressed by this natural form of deep-sea mining, Daedalus now advocates the construction of an artificial black smoker, or 'Arblasm'.

Many of the technical problems have already been solved by commercial offshore oil practice, and by the planned 'Mohole' submarine deep-drilling project. Two adjacent holes need to be drilled in the mid-oceanic sea-floor. They must reach rock so hot that entering water will dissolve it, and join the holes to form an undersea cavity. The tiniest imbalance in the two boreholes will then start a self-amplifying convective flow. One hole will develop into the downcomer of the Arblasm, delivering cold seawater into the deep cavity; the other will be its upcomer, in which superheated, mineral-saturated water will surge upwards towards the sea floor.

An Arblasm, of course, would be a wonderful tool for exploring deep sub-oceanic geology. But Daedalus reckons it could make a profit, too. He argues that the heaviest minerals, like the ores of uranium and tungsten, and the dense precious metals like platinum, gold, mercury and so on, must have sunk over geological time to the deepest layers of the Earth's crust. A properly sited Arblasm could mine them.

For this purpose, the Arblasm's exit-pipe would feed a neutrally buoyant pipe leading up to the drilling-rig mother-ship at the surface: a sort of 'chimney' for the water to rise in. In the process it would cool sufficiently to precipitate its content of precious metal as a fine suspension.

To separate the precipitated minerals, the chimney should widen towards the top, so that as the water rises, it steadily loses velocity. Each mineral will accumulate at that point in the chimney where its settling-rate matches the upward velocity. Light minerals, like copper and iron sulphides, will rise fairly high in the chimney. Denser ones like uranium pitchblende and wolframite will accumulate further down, while gold and platinum will be found near the bottom. Offtake-pipes at specific elevations in the big chimney will be able to extract these minerals in almost pure form.

David Jones

The form of pebbles

SIR—In admiring the beauty of pebbles on sea beaches, I have always suspected that they tend towards a specific form. Pebbles never approach the spherical, the two lesser dimensions never approach equality, and they are never very long (cigar-shaped). They are always somewhat flattened. The natural process of wear in the surf seems to tend to produce a certain proportion, but I have never heard of a study nor even a mention of this phenomenon. Unable to restrain my curiosity, I set out to ascertain the statistical distribution of the proportions of well-worn pebbles using a vernier caliper.

To be representative of the natural distribution, the pebbles must be randomly chosen. At the same time it is necessary to limit the choices to well-worn pebbles. I picked up samples from a large accumulation of pebbles with scarcely a glance, and examined each to see whether it could be

considered 'well worn'. If a pebble was not roughly symmetrical with respect to three planes, I discarded it. Of those accepted, I measured and recorded the three principal dimensions. Designating the dimensions in order of decreasing magnitude a , b and c , I calculated b/a and c/b .

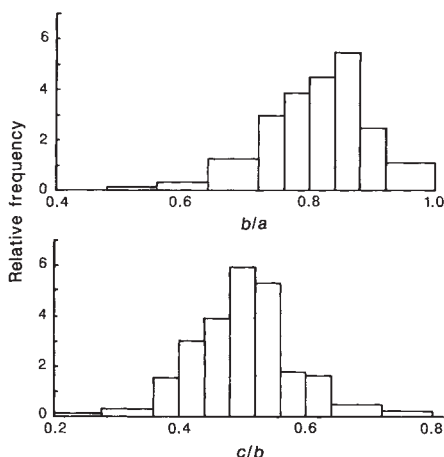
Nothing statistically significant can be concluded from the distribution of size, represented by the long dimension a , because natural transport of the pebbles along the beach by currents and by the surf results in segregation in different places by size. All the measured pebbles were between 3.2 and 12.6 cm long.

The figure shows histograms of the ratios b/a and c/b based on a sample of 200 pebbles. The mean values are $b/a=0.812$ and $c/b=0.503$. The peaks of the distributions seem to occur at about $b/a=0.86$ and $c/b=0.50$, and it is presumably to these values that the pebbles tend as they wear in the surf. Thus the ultimate stable configuration seems to be a flattened ovoid of the proportion 7:6:3 with symmetry about three perpendicular planes.

Why do pebbles tend to these proportions? The wearing process in the surf must be very complex, but somehow statistically regular. I suspect that pebbles tend not to roll at a constant angular velocity but, due to initial chance asymmetry, tend to flip. This must reinforce the inequality of the two lesser dimensions as they wear, tending eventually to produce a ratio $c/b=0.5$, which is relatively stable.

QUENTIN R. WALD

609 Trails End Road,
Wilmington,
North Carolina 28409, USA



Skua survival

SIR—Eppley and Rubega in their Scientific Correspondence¹ reported reproductive failure among South Polar skuas (*Catharacta maccormici*) nesting at Palmer Station, Antarctica, in 1988–89, and concluded that failure was an indirect effect of an oil spill from the Argentine ship *Bahia Paraíso*, which ran aground in January 1989. Although Eppley and Rubega reported no direct evidence of infection, fouling or toxicity to birds, and saw no oil on any adult birds, they nonetheless concluded that the failure must have been connected to the oil spill because of coincident timing of the two events. The authors also suggested that lapses in nest attendance, which they claimed were caused by adults attempting to clean oil from their plumage, led to the death of chicks. Here we suggest that these conclusions are incorrect.

We have studied skuas on King George Island (62°10' S, 58°30' W, 500 km north-east of Palmer station) for 10 years, and

have just completed² a 23-year study of South Polar skuas at Cape Crozier, Antarctica (77°32' S, 169°23' W). This species also failed in 1988–89 at King George Island, where there was no oil spill; the third season they have failed during the course of our studies. Similarly, in two of the three years (1982–83 and 1988–89), skuas at Palmer also failed; for the third King George failure (1986–87), no Palmer data exist. In the more severe case of Cape Crozier, the species failed in four of every five years². Breeding failure is thus hardly unprecedented for this species.

South Polar skuas coexist with the larger Brown skua (*C. lönnerbergi*) at both King George and Palmer. Where sympatric, the dominant Brown skua feeds mostly on penguin eggs and chicks and the South Polar skua feeds at sea. Breeding success among South Polar skuas at King George and Palmer³ was similar with a mean 0.70 and 0.62 chicks fledged per pair over 10 and 9 years, respectively. These datasets share five years in common, four of which are nearly identical, including the

poor 1980–81 season, productive 1983–84 year and the 1982–83 and 1988–89 years of total failure. The fifth season (1977–78) differed because persistent pack ice at Palmer³ prevented male South Polar skuas from acquiring the food needed to bring females into egg-laying condition; that season there was no ice at King George.

Poor reproductive years differ in three ways from successful ones: few pairs attempt to breed; the proportion of 1-egg (as opposed to 2-egg) clutches is high; and the fish *Pleuragramma antarcticum* is rare or absent from the skua diet. In 1988–89 at King George Island, only 9 of 33 South Polar skua pairs attempted to breed; seven of these laid only one egg, and we observed no fish fed to females during the November courtship period nor to chicks before their deaths. Similar patterns existed during the unproductive season 1982–83 at King George. This contrasts with five successful seasons during the 1980s, when South Polars laid 2-egg clutches, averaged 1.25 fledglings per pair (range 1.0–1.55), saw about half of all pairs fledge two chicks, and typically allowed tens to hundreds of intact *Pleuragramma* to lie uneaten (as surplus) at their nests.

The breeding population at Palmer was censused during 1987–88, and 306 pairs bred on five islets⁴. In 1988–89, only 88 of these pairs bred, and 81 per cent laid only one egg. These results are nearly identical to King George data, where poor food, not oil, was responsible for the reproductive failure of South Polars.

Eppley and Rubega reported that nestlings grew normally during the spill, implying that food supply was not a factor, but presented no supporting data. They reported that adults' nest-attentiveness decreased following the spill, presumably to clean oiled plumage and suggested this caused the 1988–89 breeding failure. Adult skuas typically reduce time on territory as chicks age⁵, a tendency exaggerated during food stress when chicks constantly approach to beg for more food. The parents' response is to leave in search of prey⁵ (W.Z.T. and S.G.T., unpublished data). The chicks at King George Island all died between 25 January and 15 February 1989, coincident with the post-spill period at Palmer. We conclude that lack of food caused the breeding failure in 1989 at both King George and Palmer, without help from the oil spill.

W. Z. TRIVELPIECE
D. G. AINLEY
W. R. FRASER
S. G. TRIVELPIECE

Point Reyes Bird Observatory,
Stinson Beach,
California 94970, USA

EPPLEY AND RUBEGA REPLY—Trivelpiece *et al.* contend that reproductive failure in skuas is expected whenever

small numbers of skuas attempt to breed, clutch size is small and fish is not a dominant item in the diet. In 1988–89, we found a reasonably large breeding population (163 breeding pairs censused just before clutch initiation). In a subsample of these pairs (54 nests), mean clutch size was 1.4 eggs per nest⁶. Fish was the predominant item in chick diets. We believe the differences between our values and those reported above for Palmer skuas are because Trivelpiece *et al.* started their observations later and their sampling was less frequent.

Trivelpiece *et al.* suggest that Palmer skuas would have suffered a reproductive failure in 1988–89 without the oil spill. The contrasting mortality patterns we observed in 1988–89 and 1989–90 do not support this assertion. Even though we observed complete mortality on our study sites this year, again due to intraspecific aggression, the pattern of chick mortality was very different. This year, early- and late-hatched chicks had the same life expectancy, about 2 weeks, suggesting that parents were having difficulty meeting the increasing energy demands of their growing young. Most loss occurred during storms. In the year of the spill, early-

hatched chicks had a significantly longer life expectancy than late-hatched ones⁷, reflecting the fact that virtually all chicks died within a short period coincident with the spill.

Because South Polar skuas nest at high densities and have a propensity to cannibalism, their reproductivity is sensitive to any factor which increases the likelihood of attacks by neighbours on the young. Parental neglect, rare before the spill in 1988–89, increased tenfold during the spill and affected chicks of all ages. The available evidence suggests that the mortality observed following the spill was unusual, and not due to food scarcity.

Z. A. EPPLEY

M. A. RUBEGA

Department of Ecology and Evolutionary Biology,
University of California, Irvine,
California 92717, USA

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Protein terminology tangle

SIR—The independent approaches used to identify calcium- and lipid-binding proteins, and phospholipase A₂- and blood coagulation inhibitors, means that many names are used for each member of the same protein family. A common nomenclature (see table) would improve communication between laboratories and reduce the considerable confusion that exists at the moment. The term 'annexin' which was suggested by Geisow (*FEBS Lett.* **203**, 99; 1986) because of the membrane-binding properties of these proteins, has been combined with the numbering system of Pepinsky *et al.* (*J. biol. Chem.* **263**, 10799; 1988). Forty-one

researchers have now agreed to these suggestions; we can provide a list of their names and addresses to interested readers on request.

M. J. CRUMPTON

Imperial Cancer Research Fund
Laboratories,
PO Box 123,
Lincoln's Inn Fields,
London WC2A 3PX, UK

JOHN R. DEDMAN

Department of Physiology
and Cell Biology,
University of Texas Health Science
Centre at Houston,
Texas 77225, USA

Calcium/phospholipid-binding proteins

Annexin	I	II	III	IV
Previous terminology	Lipocortin I p35 Calpactin II Chromobindin 9 GIF	Calpactin I Lipocortin II p36 Chromobindin 8 Protein I PAP-IV	Lipocortin III PAP-III 35- α Calcimedlin	Endonexin I Protein II 32.5K Calelectrin Lipocortin IV Chromobindin 4 PAP-II PP4-X 35- β Calcimedlin
Annexin	V	VI	VII	VIII
Previous terminology	PAP-I IBC Lipocortin V 35K Calelectrin Endonexin II PP4 VAC- α 35- γ Calcimedlin Calphobindin I Anchorin CII	p68, p70, 73K 67K Calelectrin Lipocortin VI Protein III Chromobindin 20 67K Calcimedlin Calphobindin II	Synexin	VAC- β

Coiling hand

SIR—John Galloway's revival in *News and Views*¹ of D'Arcy Thompson's vision of biological form being guided by physical forces is once again a reminder of just how fascinated humans are by asymmetries in a world so overwhelmingly composed of symmetrical organisms. In both *Bacillus subtilis* and the snail *Lymnaea peregra* the presence or absence of one molecule determines direction of coiling.

Regrettably, the implication that some asymmetry at the molecular level predisposes snails to coil in a particular (right-hand) direction is weakened by the results of the only other studies to examine the inheritance of coiling direction. In the polymorphic Tahitian landsnail *Partula suturalis*², as in the case of *L. peregra*³ reported by Galloway, coiling direction is controlled by a single locus. However, unlike *L. peregra*, sinistral is dominant to dextral in *P. suturalis*, suggesting that left-handedness requires the molecule whereas right-handedness does not. The pulmonate *Laciniaria biplicata* exhibits the same pattern of inheritance⁴.

Although the vast majority of living species of gastropods exhibit dextral coiling⁵, the basis of the bias remains elusive. Even more curiously, whereas gastropods are predominantly dextral, both dextral and sinistral shells were approximately equally common among conspecifically coiled fossil nautiloid and ammonoid cephalopods^{6,7}. Hence cephalopods seem to have been immune to the dextral bias so pervasive in gastropods. Perhaps differences in the early cleavage patterns, between these classes⁸ result in differential susceptibility to an intrinsic coiling bias. But then again, perhaps D'Arcy Thompson's appealing vision is just a bit too simplistic when applied to shell coiling.

A. RICHARD PALMER

Department of Zoology,
University of Alberta,
Edmonton, Alberta T6G 2E9, Canada

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GALLOWAY REPLIES—I did not mean to imply that it is some molecular asymmetry that determines hand of coiling in gastropods. In fact I emphatically think it is not. For the record, the point Palmer makes about *Partula* was made in an earlier *News and Views* article I wrote (*Nature* **330**, 203; 1987) entitled "Evolution of Helicity: Cause for Reflection".

JOHN GALLOWAY

Cancer Research Campaign,
London SW1Y 5AR, UK

Causes of mutation and Mu excision

SIR—The results reported by Mittler and Lenski¹ are completely different from ours². They find that the ara-lac fusion (which allows the Shapiro strain of *Escherichia coli* to utilize lactose when arabinose is present) occurs at an ever-increasing frequency in populations of starving bacteria in liquid medium, reaching a level of more than 10^{-6} even in the absence of arabinose and lactose. By contrast, in a large series of experiments conducted over a period of about 6 months, we could not find fusions in liquid, stationary phase cultures until arabinose and lactose were added; in our hands, the frequency of fusion after about a week of incubation at various temperatures was clearly less than 10^{-9} . Incidentally, the reason we used a rich medium for these experiments was because we did not want there to be any selection pressure for the fusion in the absence of arabinose and lactose (this strain of *E. coli* does not grow well in minimal media and, as Mittler and Lenski observed, tends to die on minimal plates whereas, for some unknown reason, it grows well and does not die once the fusion has occurred).

Several other groups have now done similar experiments. To my knowledge, one laboratory has obtained results like Mittler and Lenski's, one has found what we found and one has reported a mixture. In addition, I have now repeated our experiments using our rich medium and various minimal media, and a detailed description of these experiments was sent to Mittler and Lenski in February. I still fail to detect, in any medium lacking lactose, the presence of any bacteria that are able to form colonies on minimal-arabinose-lactose plates as fast as minority populations of cells with fusions that were introduced into these cultures at the start of the experiment.

The basis for this difference in results is not understood. Shapiro showed that the speed of production of fusions in the presence of arabinose and lactose was different for populations derived from sister colonies³ and we have found, over the years, that the ability of our standard media to allow certain re-arrangements in *E. coli* seems to be different at different times of year, apparently due to changes in the water supply. Variables like these have not been a significant complication in the study of powerful mutagens, but they give us a tantalizing glimpse of the complexity of what is, for convenience, called "spontaneous" mutation. As Shapiro wrote, "the most pertinent questions in studies of hereditary change must be questions of control and regulation", to which might be added: and the way in which these processes are influenced by circumstances.

The development of living things has

depended on variation plus natural selection. The second of these has received a huge amount of attention since the days of Darwin and Wallace, but the first has hardly been investigated at all. I can think of scarcely a dozen experiments that bear upon the circumstances of what one might call normal spontaneous mutation. And if one is interested in cancer (as I am), then surely one should be asking what are the circumstances determining this life-threatening form of somatic mutation.

JOHN CAIRNS

*Department of Cancer Biology,
Harvard School of Public Health,
665 Huntington Avenue,
Boston, Massachusetts 02115, USA*

MITTLER AND LENSKI REPLY—We stand by our results and interpretations¹. After Cairns told us of his difficulty in reproducing the accumulation of excision mutants in minimal-glucose liquid medium (without lactose or arabinose), we repeated the experiment at several different temperatures. In all cases, the frequency of mutants increased to a level significantly greater than that observed in freshly grown cells, although the relative frequency of mutants varies with temperature. J. A. Shapiro (personal communication) has also repeated our experiments, and he too has observed that "continued incubation in minimal-glucose liquid medium results in the accelerated appearance of [excision mutant] colonies on selective agar". As it was Shapiro³ who originally reported the anomalous behaviour of *E. coli* MCS2, and who provided that strain to Cairns and to us, we now have even greater confidence in our results and interpretations.

Cairns suggests that the influence of "circumstances", such as "changes in water supply", might somehow account for variation in results. Whether this is so we cannot say. However, the mere existence of variation in the results obtained with MCS2 by different laboratories does not imply that all evidence is equally credible. In particular, we must emphasize that, unlike that of Cairns *et al.*², our paper¹ included all the following essential information: (1) explicit laboratory methods; (2) experimental designs with appropriate controls; (3) the level of replication performed for each experiment; (4) the computational method used to estimate mutation rates from directly observable data; and (5) inferential statistics to guide the rejection or acceptance of competing hypotheses. Without an equally thorough accounting of such essential information, one cannot evaluate the cause of any variation between laboratories.

Moreover, we demonstrated¹ significant effects of several physiological and

population-level processes, which Cairns *et al.*² did not adequately consider. These include: (1) changes in the *per capita* rate of excision mutation with time, as cells sit starving in the absence of lactose or arabinose; (2) slight growth by MCS2 on lactose and arabinose, or trace contaminants therein; (3) cross-feeding by MCS2 due to metabolites released from Lac(Ara)⁺ excision mutants into the medium; and (4) differences in death rates between MCS2 and Lac(Ara)⁺ excision mutants. The effects of these and other similarly "mundane" processes must be taken into account in further work on the rate of excision of Mu from MCS2.

Finally, we suggest that future research should consider the role of the Mu genome⁴. Shapiro (personal communication) has shown that a Mu-encoded function is essential for most of these excisions and we have demonstrated¹ that the rate of excision increases as the host bacterium starves. A hypothesis consistent with these observations is that the Mu bacteriophage has evolved (by conventional mutation and natural selection) to use physiological cues indicative of host stress to trigger induction, of which the observed excisions may be a manifestation.

JOHN E. MITTLER

RICHARD E. LENSKI

*Department of Ecology and
Evolutionary Biology,
University of California,
Irvine, California 92717, USA*

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Radioemission source disputed

SIR—Maeda and Grebowsky¹ suggest that impulsive VLF signals observed with the Pioneer Venus Orbiter Electric Field Detector (OEFD) resemble terrestrial VLF saucers. But the similarities between the two phenomena are not as strong as they claim.

First, they use a microdensitometer scan (bandwidth 4%) of DE-1 wideband data to show that a narrow-frequency filter will result in impulsive signals. The OEFD bandwidth is 30%. Any impulse caused by a rising or falling tone will therefore last about 7 times longer than shown in their Fig. 2, corresponding to timescales of a few seconds. The bursts observed with the OEFD are known to be shorter than the decay time of the instrument electronics (~ 0.7 s)^{2,3}.

Second, although the free-energy source for VLF saucers has not been observed directly, there is strong evidence that the saucers are generated by return currents associated with the auroral cur-

ent system¹. But I am not aware of any evidence of field-aligned currents in the ionospheric holes observed at Venus.

Finally, the dispersive properties of the ionospheres at the Earth and Venus are not similar, as Maeda and Grebowsky imply. At the Earth, the sub-auroral ionosphere is characterized by relatively low electron densities and large magnetic fields, such that $f_p/f_g \sim 0.1$ (where f_p and f_g are the electron plasma frequency and gyrofrequency respectively). From cold plasma theory, the phase speed for whistler-mode waves, v , is less than $0.3c$, where c is the speed of light. In the Venus nightside ionosphere the magnetic field is weak, even within holes, whereas the density is high, so that $f_p/f_g \gg 1$, typically about 500. In this case, theory shows that $v < 10^{-3}c$. If VLF saucers were to be generated in the nightside ionosphere at Venus, their phase speeds would be even lower because VLF saucers are generated on the resonance cone, as evidenced by the V-shaped pattern in dynamic spectra¹. The phase speed would be comparable to electron thermal speeds, and the waves would be subject to electron Landau damping rather than growth.

In conclusion, there are marked differences between the observed properties of VLF saucers and VLF emissions in the nightside ionosphere of Venus. Furthermore, the ionospheres at Earth and Venus are sufficiently different that instabilities applicable at the Earth are not applicable on Venus. The saucer mechanism is therefore not as plausible an explanation for the OEFD emissions as Maeda and Grebowsky suggest.

R. J. STRANGWAY

*Institute of Geophysics and Planetary Physics,
University of California, Los Angeles,
California 90024-1567, USA*

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SIR—Maeda and Grebowsky¹ misstate the conclusions of earlier work on the source of VLF (very low-frequency) bursts in the ionosphere of Venus, ignoring the many reported properties of these bursts which may not agree with their hypothesis. It is true that, in our initial studies^{2,3}, we speculated that volcanic plumes on Venus might produce electric discharges as they do on Earth, but in the last of the three papers⁴ cited by Maeda and Grebowsky, we clearly state that “the fact that some active regions are close to, but not right over, highlands implies that volcanoes or tall mountains are probably not the immediate cause of the lightning.” We tested our hypothesis, found it wanting and rejected it.

But there are many properties of the

VLF signals that are consistent with discharges in the clouds. The rise and decay time of many of the signals seem to be smaller than the rise and decay time of the instrument⁵. These signals last for much less than 1 second, and decrease in occurrence rate with altitude at all frequencies^{6,7}. (An exception to this trend below about 150 kilometres seems to be due to refractive index changes⁸.) The signals have a strong local time asymmetry suggesting a late afternoon or early evening source^{6,7} and the Poynting flux of the waves is consistent with the expected strength of a planetary lightning source⁸.

To postulate a reasonable source for the VLF waves, one must provide a plausible energy source for the waves, explain why the energy is released in the form observed and explain the properties of the observed waves, their local time and altitude distribution, for example. The proposed plasma wave source does not yet do this. The proposed lightning source does.

C. T. RUSSELL

*Institute of Geophysics and Planetary Physics,
University of California, Los Angeles,
California 90024-1567, USA*

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MAEDA AND GREBOWSKY REPLY—We now accept that the volcanic lightning source for the Venus VLF bursts had been discarded by its advocates in a paper¹ of which we were not aware. The statement in the paper we did cite², singled out in Russell's comment, we wrongly interpreted as leaving open the possibility of such a source. For this we are in error.

But our paper was not concerned with refuting a volcanic, meteorological or any other source for lightning. Rather, we were trying to show that there are natural ionospheric emission sources which can produce whistler waves with the features seen at Venus.

We consider that the issue is still open, and offer as proof the following slight rewording of a section of Russell's comment: “There are many properties of the VLF signals which are consistent with an aurora-like emission source. The rise and decay time of many of the signals seem to be smaller than the rise and decay time of the instrument. These signals last for much less than 1 second, as would be expected for the OEFD detection of some saucer signals or other fine-scaled discrete

auroral emissions. The signals decrease in occurrence rate with altitude as would be expected for a lower ionosphere source. (The exception to this trend below 150 kilometres might be due to going beneath the source.) The signals have a strong local time asymmetry suggesting that conditions for ionospheric signal production are more favourable on the dusk side of midnight. The electric field energy density in the 100-Hz waves is the same order as the E field energy density in typical terrestrial saucer emissions.”

Strangeway's comment focuses on evaluating the VLF saucer production hypothesis within the Venusian environment and has some merit. The discussion of the potential importance of Landau damping may indeed rule out one of the two mechanisms for saucer production cited in our paper. But the point that discrete saucer-like emissions can produce emissions of less than 1-second duration as observed from the Pioneer Venus OEFD is still valid. Although our densitometer analysis of terrestrial DE-1 data used a narrower bandwidth than the Pioneer instrument, one cannot simply scale the durations as Strangeway has done — the scaling depends on the aperture angle of the saucer ray in the frequency–time spectrum. If the source region, for example, is traversed, the duration is the same regardless of the detector bandwidth.

The most telling argument against an ionospheric plasma source would be an analysis of the VLF Venus bursts that showed that the bursts were all consistent with durations less than the instrument resolution time. Unfortunately, the presence or absence of whistler bursts of duration longer than the instrument sample time has yet to be determined. In addition, Strangeway points out that there is, as yet, no evidence for field-aligned currents in the Venus holes but at the base of the nightside ionosphere the high-resolution B-field measurements show extreme variability¹, thus providing evidence for the existence of plasma current sheets.

Within the framework of the information known or surmised about VLF bursts and the local plasma environment, we therefore hold that a local ionospheric source is still a viable hypothesis. To resolve these issues, proper observational data of the Venusian nightside VLF emissions, such as the broadband frequency–time spectrograms, must be obtained instead of repeating speculative arguments based on the fragmentary monochromatic data.

K. MAEDA

J. M. GREBOWSKY

*NASA/Goddard Space Flight Center,
Greenbelt,
Maryland 20771, USA*

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Merit of the children

John C. Marshall

Legacy of Silence: Encounters with Children of the Third Reich. By Dan Bar-On. Harvard University Press: 1989. Pp.338. \$25, £22.

DR DAN BAR-ON is a psychologist who teaches at the University of the Negev in Israel. His parents were German Jews who lived in Hamburg, and the family had been well-established in Germany for more than two centuries. Most members of the family were, of course, sincere German patriots. Bar-On's grandfather had served as a doctor in the German army and "liked his uniform so much that he even wore it at his wedding". Bar-On's father, also an assimilated physician in a working-class district of Hamburg, listened to Josef Goebbels's announcement of the Jewish boycott in April 1933, read the pulse of the times accurately, and with his wife and little boy set sail for Palestine. Most members of the family who remained in Germany were made into lampshades, fertilizer and bars of soap.

For the next fifty years, Bar-On lived among survivors and the children of survivors until sometime between 1985 and 1987 when he returned to Germany in an attempt to understand the children of the perpetrators. *Legacy of Silence* is a record of Bar-On's interviews with the (now adult) children of men whose fathers ranged from minor functionaries of the Holocaust to serious mass-murderers. The book would demand to be read at any time but must certainly be read now at a time when the whole of Europe (and its colonial outposts) is in a state of singular ferment. The truth may, or may not, make us free, but repression of the past will most certainly make our societies psychotic.

That six million people were killed for no other reason than that they were 'Jews' (whatever that means) remains the central datum of European history in this century. Revisionist historians point out (and correctly so) that the Nazis have held no monopoly on mass-murder in this (or any other) era, and that Jews have never been the sole victims of racial, religious or ideological terror. Yet all these ways of relativizing the number of dead seem little more than an obscene excuse to avoid confronting what happened within a country whose standards of education and culture had reached unparalleled heights.

All manner of 'explanations' — economic, psychological and theological — have been attempted and we still understand nothing. Rich and poor, peasant and professor, atheist and Christian alike kept the gas chambers running . . . just as a few members of every imaginable group protected the lives of others, often at the

cost of their own. If the point of any enlightened philosophy is to understand the past with a view to creating a more

close to total honesty as it is humanly possible to achieve. And such is his skill as an interviewer that one feels his partners in dialogue have likewise reached the limits of bearable insight into the mentality of their elders.

The sons and daughters are as disparate as the fathers. The doctor in a small Tyrolean village reminds Bar-On of his own father, but this father had been a physician at Auschwitz, although acquitted of any crime "because inmates testified

IMAGE
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humane future, we still live in the dark ages.

Bar-On's attempt wisely avoids the pathetic banalities of biological, psychological and sociological 'theories' as they are typically applied to issues that concern our humanity (and its perversions). Rather, he reports his conversations more or less verbatim without trying to cover up the pain, anger and silence of both interviewee and interviewer. These reports are then interwoven with Bar-On's extended reflections on his own feelings and state of mind. His account probably comes as

about his helpful and humane behaviour toward them". His son Peter 'knows' the history of the father and the father's generation but is comfortably resigned to the recurrence of evil. Manfred's father (who he never knew) was a high-ranking scientist involved in the 'euthanasia' program who committed suicide after the war; Manfred, a professor of history, is trying to understand his unknown father and worries "if he is normal because he does not have nightmares like the children of other perpetrators". Helmuth's father worked for the Racial Hygiene Committee

and was a local director of 'Health Services'. Helmuth is a television producer and writes poetry. He cries when he describes how, in a clinic at the University, his father shot himself. As a child Helmuth was convinced that his mother wanted to poison him; he cannot now listen to those beautiful folk songs (*Klein schönes Land*) with which his father sang him to sleep.

Hilde grew up in the camps where her father was a 'supplier'. Her predominant memory, only now surfacing, is that "they used to call me 'camp doll' all the time, the people in the kitchen and the women at the parties". Thomas, the nephew of Reinhard Heydrich, is an actor who performs cabaret songs of the 1920s and 30s in the local theatres. He specializes in the songs of the German-Jewish poets Tucholsky and Mehring; he wants to go on a reading tour of Israel, reading Heine in the original German. The thought that goes through Bar-On's mind is "they'd kill him". But he does not say this to Thomas, and over lunch he finds himself telling Heydrich's nephew about Lurianic cabalism ("within each evil you find a sign for good"). Thomas in turn tells Bar-On of his girlfriend's brother who converted to Judaism and became an orthodox rabbi.

In the Old City of Jerusalem, Bar-On meets this man (now called Menachem), the son of a prominent member of the SS. With beard, gabardine and kipa, he "teaches Jewish theology and philosophy to Israeli students" at Tel Aviv University. Returning briefly to Germany for his father's funeral, he can feel nothing.

In the concluding chapter, Bar-On suspects he should "analyze his data", but realizes that the point is rather to confront it. Perhaps the third and fourth generations will see more clearly than we do. Or will Bar-On's children be putting similar questions to the children of Ariel Sharon and Meir Kahane? Nearer to home, the right honourable Mr Norman Tebbit tells us that the criterion for the assimilation of the terrified British residents of Hong Kong into the country whose passports they hold is "which side do they cheer for?". As a British subject with a white skin, albeit, as we used to say, of the "Mosaic persuasion", I had not realized that my continued citizenship of the United Kingdom was dependent upon my active support of the English cricket team (nor that Peking had a cricket team).

Jewish doctrine teaches a counterpart to the 'merit of the fathers' (conspicuously lacking in the fathers described by Dan Bar-On). It is the notion that the righteousness of the living child favourably affects the fate of the dead father. Some of us may need this "merit of the children". □

John C. Marshall is in the Neuropsychology Unit, part of the Neuroscience Group at the Radcliffe Infirmary, Oxford OX2 6HE, UK.

Slipping from the heights

David J. Prior

Mountain Environments. By A. J. Gerrard. *Belhaven: 1990. Pp.317. £30.*

THERE are few subjects within Earth studies with greater potential for an illuminating and exciting discussion of the interaction of competing geological, geomorphological, biological and human processes than mountain environments. A. J. Gerrard makes a bold attempt to summarize the current understanding of the processes influencing the environmental patterns in the Earth's mountains. The range of source material used is impressive and the bibliography is an invaluable data bank, but I feel the author could have discarded much of the outdated work and attempted to reconcile various approaches and models, even if this had meant imprinting some of his own bias.

Gerrard is preoccupied with semantics and, after reading his book, one has to question the validity and usefulness of simple categorization of complex natural phenomena. Much of Gerrard's discussion revolves around the intricacies of nomenclature: this approach is taken to an extreme in a discussion of the definition of mountains in the early part of the book. When does a hill become a mountain and a mountain a 'high mountain'? Such a distinction is subjective and any discussion thereof is as intractable as it is pointless. Gerrard also subjects processes to discriminatory nomenclature. Does it matter whether a landslide event is classified as a rockfall, a rockslide, a debris slide or a debris flow? Of course there are compet-

ing processes which contribute to the character of a landslide, but what they are called is largely irrelevant. The study of mountains is predominantly an observational science, and a plethora of processes contribute to mountain environments. The construction of any rigid classification of such complex phenomena limits our ability to analyse them objectively as preconception is often inadvertently built into the classification scheme. A better grasp of mountain environments and their controlling processes will be achieved only by detailed quantitative analysis of the interactions of processes in specific natural examples.

Gerrard clearly appreciates the interdisciplinary nature of his topic, and covers diverse aspects of Earth sciences, biology and social sciences. The natural examples essential for the data source of a review of mountain environments are scattered liberally through the text with little underlying context, and local geographical names are freely used without map reference. The introductory and concluding chapters are vague, verbose and poorly argued. Chapters describing the surface processes which influence mountain morphology comprise the most coherent section of the book. Each of these review chapters is illustrated with excellent synoptic diagrams and tables extracted from the scientific literature. In a chapter on mountain geocology, Gerrard presents a useful set of data, again mostly in diagrams, but the text is little more than a series of mundane lists that merely duplicates the information in the figures. A chapter on volcanoes as mountains is inappropriate without coverage of the geological controls on non-volcanic mountains in equivalent detail.

A discussion of the potential tectonic controls on mountain development, for which there is now a considerable literature base, is crucial to any treatise on mountain environments, and it is sad that this aspect is ignored in this book. As a geologist, I am alarmed by the misconception of many principles of mechanical behaviour of Earth materials shown by the author and this points to the main failing of this book: the topic of mountain environments is too broad to be covered by one author. The interdisciplinary nature of the subject necessitates that any comprehensive review must consist of a volume of papers written by specialists. □

David J. Prior is in the Department of Earth Sciences, Liverpool University, Liverpool L69 3BX, UK.

■ Still in the hills, *The Magnificent Mountain Women* by Janet Robertson, charts the multifarious exploits of women who have ventured into the Colorado Rockies from the 1850s to the present day. Published by University of Nebraska Press \$28.25. To be published in Britain 30 June, £20.85.

New Journals

This year *Nature's* annual new journals review supplement will appear in the issue of 11 October. Publishers and learned societies are invited to submit journals for review, taking note of the following criteria:

- Journals which first appeared after June 1988, and which issued at least four separate numbers by the end of April 1990, will be considered for review. The deadline for submission is the end of June.
- Journals covering any aspect of science are eligible, although those dealing with clinical medicine, engineering and pure mathematics are excluded, as are publications of abstracts.
- Frequency of publication must be at least three times a year.
- The main language used must be English. Translation journals in English are eligible.

When submitting journals for review, please send at least four different issues (the first, the most recent and any two others) of each title as soon as possible to: Book Review Editor, *Nature*, 4 Little Essex Street, London WC2R 3LF, UK or 1137 National Press Building, Washington DC 20045, USA.

Big science by design

Bernard F. Burke

Astronomer by Chance. By Bernard L. Lovell. *Basic Books*: 1990. Pp.358. \$24.95. To be published in Britain by Macmillan.

IT WAS sunny and warm in Cheshire, that August of 1955, when the radio astronomers of the world gathered at the Jodrell Bank Experimental Station for a symposium on radioastronomy, one of the earliest symposia held by the International Astronomical Union. Professor Bernard Lovell was our host. Through the window of the side wall of the lecture hall we could see the scaffolding and steel works of the unfinished 250-foot telescope, and during lunchtime breaks and at dinner the talk often turned to that project. Much of the time, our opinions were not complimentary. Galactic astronomy was being completely remade by the 21-centimetre hydrogen line results from groups at the Leiden Observatory and CSIRO Division of Radiophysics, whose presentations at the symposium were polished and impressive. Steerable paraboloids, clearly, had to have far tighter surface tolerances than the 250-foot telescope was designed for if they were to meet the 21-centimetre challenge. The 250-foot telescope had been redesigned, the original mesh to be replaced by a solid surface, but the general opinion among the younger radioastronomers was not favourable.

Not only did it seem to be inadequate by 21-centimetre work, but there were other intellectual currents that did not seem to favour a 250-foot dish. Interferometric arrays were becoming widely used, and the Jodrell astronomers had been at the forefront of the new developments. The study of extragalactic continuum sources was one of the hottest topics at the time. At the symposium there was a spectacular clash over the statistics of radio sources, between the Cavendish group at Cambridge, led by Martin Ryle, and the Australian Mills Cross group at CSIRO, with Joe Pawsey as spokesman, backed up by Bondi, Gold and Hoyle, who vigorously defended the steady-state cosmology against Ryle's attack. This was heady stuff for the young radioastronomers, and the 250-foot telescope seemed to be clearly out of the scientific mainstream. We were not aware of the deeper troubles that were preoccupying Lovell, and not even he could have known that the troubles with labour, cost escalation, and redesign were only preludes to the grievous times that lay ahead. The course of forefront research seldom runs smoothly,

but the threat of imprisonment is not usually among the hazards of scientific life.

Sir Bernard's memoir has the Jodrell Bank telescope drama as a central theme, but it is set into his life in a graceful way. He tells about his journey from a small village in the west country, to Tyndall's laboratory in Bristol, and from there to Manchester under Blackett. The journey was broken by the war, and his personal story of the development of the H_2S radar should be a useful supplement to the more complete accounts given by "Taffy" Bowen and Henry Guerlac. There was,

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Thanking his lucky stars — Sir Bernard Lovell with the 'big dish' of the radiotelescope at Jodrell Bank.

certainly, a transatlantic rivalry, and Sir Bernard's view gives a good account of Britain's pioneering role. The British and US histories will each have their national bias, but it was the British physicists who stood in the breach alone at the most critical time, and Sir Bernard was one of the warriors in that struggle. He is aware of the good and evil that resulted, as the same radar that guided the pathfinders in laying out their patterns of destruction on the German cities also served to suppress the German submarine menace in the Bay of Biscay.

The story of the transition to peacetime is told well. The way was hardly smooth, as Sir Bernard's research effort had to start from nearly nothing except the good will of Blackett and the telephone numbers of some old comrades in the defence establishment. In the early years, life at Jodrell Bank was very much the same as

that at the Cavendish, setting up hand-made lash-ups and surplus wartime military gear in the mud of borrowed fields. The evolution took divergent paths towards the same goal. Martin Ryle's style at the Cavendish, started with studies of solar radio noise and evolved towards the study of extragalactic sources, using clever antenna concepts at minimum expense, with emphasis on interferometry and the use of Fourier transforms — an old Cavendish tradition. The Jodrell Bank programme, on the other hand, started with radar as the operating tool, and

sometimes with radar there is no inexpensive but clever fix. I think that this reality accounted for much of the difference in style that evolved at the two laboratories. Radar needs high-power modulators and transmitters, heavy hardware, and as much antenna area as possible. Paraboloids are a natural choice, and the philosophical drive of radar led to the Jodrell Bank 250-foot paraboloid as a logical development.

An interesting contrast in research philosophy underlaid our discussions in those days, unrecognized but insistent. At the end of the Second World War, radioastronomy in the United States received little attention. There was a lot of money around once the Office of Naval Research and its counterparts in other services were established, and while laboratory physics flourished, radioastronomy was neglected. In the United Kingdom and Australia, the scale of laboratory support was far more modest, and the groups at Manchester, Cambridge and Sydney had to start on a shoe-

string, with occasional moral encouragement in high places as their principal capital. The plentiful store of surplus radio gear made radioastronomy a natural choice. As the new field grew, we knew first-hand the thrill of discovery with equipment that had been assembled with our own labour, and that shared experience was a bond. None of us, I think, including Bernard Lovell, appreciated the symbolism of that rising steel framework, which foreshadowed the end of a research style.

A few years later, Martin Ryle would formulate aperture synthesis and the Cavendish laboratory would be buying three US radiotelescopes for the one-mile telescope array, and Jodrell Bank would be spreading paraboloidal radiotelescopes across the countryside for the MERLIN array. Interferometry was the wave of the future, but large paraboloids would be the key elements. The 250-foot telescope

would eventually be brought into modern functional form in 1970–71, and it still serves today as a vital part of the world-wide VLBI network. Sir Bernard tells an interesting story of how this rebuilding came about, when he was made chairman of the Astronomy, Space and Radio Board of the UK Science Research Council, as it was then called. The administration of the university was looking forward to the rich rewards that he could reap with his high post, but he chose to set aside his dreams of a far larger dish in favour of Martin Ryle's new one-mile aperture-synthesis telescope. The future development from that point moved inexorably away from the hands-on personal experience to large, professionally tended facilities. Radioastronomers, with Jodrell Bank in the lead, had made the old lifestyle redundant.

Sir Bernard's memoir is relatively brief, but it is beautifully written and easily understood by a lay reader. My wife greatly enjoyed his telling of how Joyce Lovell played a crucial role in improving the quality of her husband's 1958 Reith lectures. The title of the book is apt, and serves as a reminder for many of us how subject our decisions are to chance events. Without the fortunate timing of the Sputnik by the Soviet Union, Sir Bernard would have been in serious trouble, but in October 1957, he became a hero instead. He wears his laurels modestly, and gives credit to the talented young people who, under his leadership, made Jodrell Bank such an interesting place.

At the first reading, I wished for more description of the inside workings of the system, but that is not his style. The monumental clash between Ryle, Pawsey and the Cambridge cosmologists is not mentioned; in fact, there is no mention of the symposium itself, despite Jodrell's central role. In a more gossipy vein, it would have been fascinating to learn more about the dealings between Sir Martin and Sir Bernard over budget priorities, which must have been intense at times. In retrospect, these omissions are entirely in character. In the summer of 1955, solving the enormous financial and engineering problems were far more vital to Jodrell Bank's future than a prestige item like a symposium. Sir Bernard always attended to first things first. The lack of gossip is also consistent, because he is a gentleman, and gentlemen have no need of gossip. He credits Sir Martin with the same virtue; knocking the other man's project is no way to further one's own interests. Sir Bernard's consistency has served him well, and astronomy as a whole has been well-served at the same time. □

Bernard F. Burke is in the Department of Physics, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA.

Essential link

Geoffrey Tweedale

Early Scientific Computing in Britain. By Mary Croarken. *Oxford Science Publications*: 1990. £25.

UNTIL the 1950s, computing was regarded as a highly esoteric activity. Even the great von Neumann regarded powerful computers as the tool of applied scientists — quite exclusively. It was also believed that computing would be a highly centralized activity, with only a handful of centres providing the facility for solving complex calculations for scientists, engineers and surveyors.

Croarken takes as her main theme the gradual centralization of computing power in the United Kingdom during the first half of the twentieth century. Although the development of desk calculating machines in the late nineteenth century made it theoretically possible to centralize some types of scientific computing, this trend only began to emerge in the late 1920s. A key figure was L. J. Comrie, a New Zealand-born astronomer, who joined the Nautical Almanac Office in 1925 and soon became its superintendent. Under Comrie, the NAO mechanized much of its computation and developed efficient numerical methods for use with mechanical computing aids, such as Hollerith punched-card machines. The NAO was thus transformed from rather a narrow institution, providing navigational data, to a world-renowned centre involved in astronomical societies, tablemaking and mechanical computation generally. In other words, it had come close to becoming an unofficial computing centre. A driven and impatient man, Comrie eventually found the civil service environment of the Admiralty uncongenial and in 1937 he left to set up his own firm, the Scientific Computing Service. By the beginning of the Second World War Comrie's London-based business had become the only commercially run scientific and computing service to exist in Britain (and probably the world).

Meanwhile, mathematicians at Manchester and Cambridge universities, finding that Hollerith punched-card machinery and desk calculators were inadequate for their needs, began the construction of large-scale analogue machines. In 1934 Hartree, a professor of mathematics at Manchester University, constructed a differential analyser. Although useful for complex differential equations, such as those in ballistics, the analyser did not lead to the growth of a computing centre at Manchester University. However, Hartree's work did provide a springboard for later work on digital computers both at Manchester and at

Cambridge, where another differential analyser was built and the Cambridge University Mathematical Laboratory was founded in 1937.

Nevertheless, when the Second World War broke out the only organization to which scientific workers, both within and outside government, could apply for computational advice or practical assistance was Comrie's Scientific Computing Service. The sudden upsurge in demand for computing services from the military thus created a problem. Initially, the expertise and resources of individuals such as Comrie and Hartree were used and computation within the government's service ministries was expanded. The Admiralty Computing Service was established in 1943, though its role as a central computing service was limited by its restriction to government work, its lack of equipment and failure to undertake new research. These deficiencies were eventually recognized, leading to a proposal from the Department of Scientific and Industrial Research for the creation of a genuine national computing centre — the National Physical Laboratory Mathematics Division — which became operational by 1946. By then the electronic stored-program computer was about to make its entrance, leading first to the development of other computing centres in the 1950s, but eventually to the decentralization of computing services.

The author's investigation of scientific computation in the 1920s and 1930s is an important contribution to the history of computing, though the final sections of the book are something of a disappointment: the discussions of the work of the NPL and the post-war development of computing at Manchester and Cambridge add little to previous accounts. Croarken seems to avoid the wider implications of her work: there are no international comparisons and little analysis of the social and economic context. She argues that the "most important single factor which prompted the creation of computing centres was the work of far-sighted individuals" — yet the fact that the foundation of a centralized computing service was undertaken only through the pressure of war makes such a conclusion seem simplistic.

Nevertheless, Croarken reminds us that, despite the enormous strides of the past forty years, calculating machines and computers have had a long history. Though mostly using only published secondary sources, she sheds a clear and illuminating light on early pioneers such as Comrie and Hartree and provides an essential link between their work and that of their more famous heirs from the electronic digital era. □

Geoffrey Tweedale is at the National Archive for the History of Computing, Manchester University, Manchester M13 9PL, UK.

Global climate change and US agriculture

Richard M. Adams*, **Cynthia Rosenzweig†**, **Robert M. Peart‡**, **Joe T. Ritchie§**,
Bruce A. McCarl||, **J. David Glycer¶**, **R. Bruce Curry‡**, **James W. Jones‡**,
Kenneth J. Boote** & **L. Hartwell Allen, Jr.††**

* Department of Agricultural and Resource Economics, Oregon State University, Corvallis, Oregon 97331, USA

† Goddard Institute for Space Studies, Columbia University, New York, New York 10025, USA

‡ Department of Agricultural Engineering, University of Florida, Gainesville, Florida 32601, USA

§ Department of Crops and Soil Science, Michigan State University, East Lansing, Michigan 48824, USA

|| Department of Agricultural Economics, Texas A&M University, College Station, Texas 77843, USA

¶ Christensen and Associates, 4610 University Ave., Madison, Wisconsin 53705, USA

** Department of Agronomy, University of Florida, Gainesville, Florida 32601, USA

†† USDA-ARS, Department of Agronomy, University of Florida, Gainesville, Florida 32601, USA

Agricultural productivity is expected to be sensitive to global climate change. Models from atmospheric science, plant science and agricultural economics are linked to explore this sensitivity. Although the results depend on the severity of climate change and the compensating effects of carbon dioxide on crop yields, the simulation suggests that irrigated acreage will expand and regional patterns of US agriculture will shift. The impact on the US economy strongly depends on which climate model is used.

CARBON dioxide concentration of the atmosphere has increased from ~280 p.p.m. before the industrial revolution to ~350 p.p.m. today¹. According to most climate models, a continued build-up of CO₂ and other infrared-absorbing 'greenhouse' trace gases is likely to lead to surface air temperature rises of 1.5–5.5 °C and changes in precipitation patterns over the next 50–75 years^{2–7}. Climate changes of the magnitude suggested by climate models will have agricultural consequences. Many authors have presented qualitative discussions on these agricultural implications^{8–10}, but quantitative estimates of changes in crop yields and irrigation water use and the resulting effects on producers and consumers have not appeared.

Here we report the results of a multidisciplinary study using global climate models, crop-growth models and an economic model to estimate effects of possible CO₂-induced climate change on US agriculture. Although this research provides quantitative estimates of potential effects on the agricultural economy, perhaps the main contribution is highlighting uncertainties in current knowledge. For example, differences in average seasonal temperature and precipitation between two climate model forecasts result in substantial differences in crop yields and irrigation water requirements in some regions of the US. The effect of these and other uncertainties on the economic assessment is one means of finding priorities for future research.

Another contribution is recognition of the value of integrating models from various disciplines, here atmospheric science,

agronomy and economics. Although interdisciplinary efforts of this kind are encouraged^{11–13}, it is stressed that the results of such an integrated approach do not predict the future; rather, evaluation of results offers insights into effects of the hypothesized conditions (here, CO₂-induced climate change) on a system (here, the US agricultural economy) as it is currently understood. These insights may then contribute to the larger societal policy discussion of both potential control strategies to reduce CO₂ and other trace-gas emissions and on appropriate strategies to prepare for change.

Effect of CO₂ increases on US climate

A number of global-climate research projects have calculated likely climate changes under alternative CO₂ levels using numerical models of the atmospheric general circulation known as GCMs⁷. The forecasts used here are from the NASA/Goddard Institute of Space Studies (GISS) model¹⁴ and the Princeton Geophysical Fluid Dynamics Laboratory (GFDL) model⁵. Ratios of mean monthly temperature, precipitation, and incident solar radiation for doubled CO₂ (to 630 and 600 p.p.m. respectively) simulations to current climate simulations were applied to observed (1951–1980) daily station climate variables. Observed variables at individual locations were multiplied by ratios of climate change from the appropriate GCM gridbox (8° lat. × 10° long. for GISS; 4.5° lat. × 5° long. for GFDL). No interpolations were made between or within grid boxes because GCM calculations do not account for this variation. The calculation of the GISS and GFDL climate-change models does not

TABLE 2 Derived climate parameters of selected agricultural regions as predicted by GISS and GFDL

Region*	Evaporation ratio†		Precipitation ratio†		Average annual temperature increase (°C)	
	GISS	GFDL	GISS	GFDL	GISS	GFDL
Southeast	1.08	0.93	1.11	0.92	3.5	4.9
Delta	1.02	1.02	1.02	1.00	5.3	4.4
Northern Plains	1.09	0.99	1.07	0.97	4.7	5.9
Southern Plains	0.99	1.02	0.92	1.00	4.4	4.5
Mountain	1.10	1.06	1.11	0.99	4.9	5.3
Pacific	1.11	1.03	1.15	1.02	4.7	4.7

Source: ref. 30.

* The southeast region includes Florida, Georgia, South Carolina and Alabama; Delta region includes Arkansas, Louisiana and Mississippi; Northern Plains are Kansas, Nebraska, North Dakota and South Dakota; Southern Plains are Oklahoma and Texas; Mountain region includes Arizona, Colorado, Idaho, Montana, New Mexico, Nevada, Utah and Wyoming; Pacific region includes California, Oregon and Washington.

† Evaporation ratio is the doubled-CO₂ forecast for evaporation relative to the current-CO₂ forecast.

‡ Precipitation ratio is the doubled-CO₂ forecast for precipitation relative to the current-CO₂ forecast.

TABLE 1 Summary of climate change in GCMs for doubled CO₂: average of US grid points

Model	Temperature change (°C)			Precipitation change (mm day ⁻¹)		
	Annual	Winter	Summer	Annual	Winter	Summer
GISS	+4.32	+5.46	+3.50	+0.20	+0.13	+0.24
GFDL	+5.09	+5.25	+4.95	+0.09	+0.19	−0.08

Source: ref. 30.

include changes in the space and time distribution of climate events. Therefore many significant climate and biophysical features are ignored. Forecasts of annual as well as seasonal temperature and precipitation changes are summarized in Tables 1 and 2. The use of two GCM models reflects some of the variation in climate estimates. For example, both models forecast increases in regional temperature but differ on changes in precipitation and evaporation. Table 2 points out the lack of consensus for some important agricultural regions, an issue that has implications for assessing climate-change effects^{7,15}.

It should be stressed that the doubled-CO₂ model represents an equilibrium climate, rather than one responding transiently to the gradually increasing trace-gas forcing. Because other trace greenhouse gases (for example, CH₄, N₂O, chlorofluorocarbons) are increasing, the climate-change models may be considered to be an 'effective' doubling of CO₂, meaning that the radiative forcing of all greenhouse gases has the same radiative forcing as doubled CO₂. The effective doubling of CO₂ concentrations will occur around the year 2030, if present emission trends continue⁴. The climate change caused by this effective doubling may be delayed, owing to slow uptake of heat by the oceans and other factors. A more detailed discussion of these and other uncertainties is presented by Schneider⁷.

Physical effects on the agricultural system

Crop yield effects. The climate changes described in Tables 1 and 2 are expected to lead to changes in crop yields, but substantial resources and time would be needed to perform experiments to explore even part of the range of these variations. In this study, the SOYGRO (version 5.41)¹⁶, CERES-Maize¹⁷ and CERES-Wheat¹⁸ dynamic-growth crop-simulation models are used to project the effects of climate changes on the yields of irrigated and rainfed winter wheat, maize (corn) and soybeans^{19–21}. The choice of these widely validated models is based on several criteria. First, the models simulate crop response to the major climate variables of temperature, precipitation and solar radiation, and include the effects of soil characteristics on water availability for crop growth. They are also physiologically oriented, with functions that calculate the rates of photosynthesis, translocation, respiration and other crop processes. Growing-degree days and photoperiod effects are modelled for the three crops. Second, they are validated for a range of soil and climate conditions. Third, these models are developed with compatible data structures so that the same soil and climate data bases could be used with all crops. An important issue in assessing agricultural effects of climate change is the role of elevated CO₂ on plant growth^{22,23}. Increased CO₂ has been found to increase photosynthesis and decrease stomatal conductance in most crop plants in experimental settings, resulting in reduced transpiration rate per unit leaf area and overall increase in water-use efficiency (seed yield divided by total evapotranspiration during the growing season (kg ha⁻¹ mm⁻¹))^{23,24}. Modifications to the crop models were made to calculate photosynthesis and evapotranspiration rates under increased CO₂^{19–21}. Photosynthetic rates were increased by 35, 25 and 10% for soybeans, wheat and maize, respectively, under doubled-CO₂ conditions.

Limitations of the crop models. Although the crop-simulation models were developed and tested over a range of climate conditions, they have not been tested under the temperature conditions suggested by the GCMs. The crop models also assume that soil nutrients are not limiting, there are no other major soil problems, and that pests (insects, diseases, weeds) pose no limitation to crop growth and yield. Further, the beneficial effects of CO₂ on crop yields will be overestimated if the equivalent warming of the doubled-CO₂ climate occurs before an actual doubling of atmospheric CO₂ (due to increases of other greenhouse gases) and if the experimental results are not reproduced under warmer, more variable and pest-infected field conditions. Finally, changes in climate variability, which

TABLE 3 Predicted percentage change in irrigated-crop water use requirements and water supply for irrigated regions*

Region	Irrigated-crop water use		Supply†‡	
	GISS	GFDL	GISS	GFDL
Southeast	15	147	16.0	-9.0
Delta	16	60	2.0	-3.0
Northern Plains	-13	14	1.0	-3.0
Southern Plains	-4	-10	-3.0	-2.0
Mountain	-8	2	2.0	-7.0
Pacific	-9	3	7.0	-2.0

* Regions are defined in Table 2.

† Water availability in areas that currently do not rely on irrigation is not addressed in these analyses. These excluded areas contain <3% of current (1985) irrigated acreage.

‡ Changes in water supply are calculated using base weather, precipitation (ratios) and evapotranspiration (ratios). Specifically, the percentage change in water supply is calculated as $\% \Delta(WS) = [(BP \times PR) - (BE \times ER) - BWS] / BWS \times 100$ where WS, water supply; BP, base precipitation; PR, precipitation ratio (forecast precipitation/base precipitation); BE, base evaporation; ER, evapotranspiration ratio (forecast evaporation/base evaporation); and BWS, base water supply (BP - BE).

were not simulated, could significantly alter the results. If frequency of droughts or mesoscale convective complex (MCC) rainfall and hail damage increases, climate-change effects on crop yields could be more severe²⁵.

Crop yield results. Rainfed-crop yield estimates shown in Fig. 1 differ markedly for the two GCMs, even though the annual average changes shown in Table 1 are not very different. Crops are sensitive to weather over relatively short periods of time, and annual averages do not convey important shorter-term differences. For example, consider the average monthly growing season precipitation for one location, Columbia, South Carolina. The historical average for June is 113 mm. For the doubled-CO₂ analysis, the models differ sharply in monthly precipitation forecasts, with GISS forecasting 148 mm and GFDL 50 mm. The lower summer growing-season precipitation for the GFDL model accounts for its much lower yields under rainfed conditions.

The simulated changes in crop yields are driven by two effects, changes in climate and CO₂ enrichment. The interaction of these forces leads to predictions that vary by region. In more northern latitudes a longer frost-free growing season and increased temperatures had a beneficial effect on simulated yields. In other regions, high temperatures shortened the duration of crop growth stages, especially grain-fill, resulting in moderate to severe yield decreases^{19–21}. The beneficial effects of elevated CO₂ on crop yields, however, offsets some or all of the adverse climate effects. As a result, average yields in most regions actually increase in the milder GISS climate-change model, whereas a pattern of yield decreases persists in the hotter and drier GFDL model. Irrigated yields tend to be higher and less variable than rainfed yields, in both the base-period and climate-change scenarios^{19–21}.

In regions where present crop yields are relatively low, high percentage increases in yields do not indicate large absolute increases in crop yields. For instance, the Lake States and Northern Plains regions currently have low relative average yields based on 1951–1980 climate, so these increases may not be as significant as the smaller percentage increases in the higher-yielding Corn Belt. Similarly, for the Southern Plains, Mountain, and Pacific regions, changes in maize yields for rainfed production are not as important as changes under irrigation, because the majority of the production from these regions is from irrigated land.

Irrigation water use and availability. Rising temperatures in Tables 1 and 2 suggest increased evapotranspiration, crop water use and irrigation demand. The CERES and SOYGRO models

calculate the irrigation water applied by irrigating the soil profile up to field capacity whenever the crop becomes water-stressed. Changes in irrigated-crop water-use requirements are presented in Table 3. The irrigation water use values reported in Table 3 for the GISS and GFDL models are used to adjust irrigation water requirements in the economic model of the agricultural sector. Changes in water use vary with GCM models, with modest increases or slight decreases under GISS but substantial increases projected under GFDL in the southern United States.

Changes in ground- and surface-water availability are estimated using a simple hydrologic mass-balance approach that reflects the interactions of evaporation, rainfall and temperature forecasts from the GCMs (Table 3). If a region currently has greater annual precipitation than annual potential evaporation, the same percentage increase in evaporation and precipitation could increase the differential, suggesting an increase in runoff. Predicted changes in regional water supply that reflect these estimates are reported in Table 3. Note that GFDL projections are much more severe than GISS, showing water-supply reductions in all irrigated regions, caused by increased evaporation driven by high temperatures. The procedure used to calculate regional changes are described in Table 3. Assumptions underlying forecast changes in agricultural water availability are discussed by Adams, Glycer and McCarl²⁶.

Economic consequences. The final step in the assessment involves adjustments in the parameters of an economic model of the US agricultural sector to reflect the crop yield, water use and water-supply effects of climate change. A correct translation of these effects into their implications for societal well-being requires a model that reflects the adjustments that producers, consumers and other affected parties are likely to take to soften the impacts of adverse climate¹². Thus, human behavioural responses to adverse climate must be modelled.

The economic model is one previously used in a number of appraisals²⁷⁻²⁹ and is designed to simulate the effects of changes in agricultural demand, resource usage or resource availability on agricultural prices, quantities produced, consumers' and producers' welfare, exports, imports and food processing. The model considers production, processing, domestic consumption, imports, exports and input procurement for most crop and livestock commodities. Included are both primary commodities (those produced directly by farms) and secondary commodities (activities such as soybean crushing and livestock feeding and processing).

A total of 1,683 production possibilities are specified to represent major field crop and livestock production in the United States. For some regions, field crops are divided into irrigated and non-irrigated production. Each crop and livestock

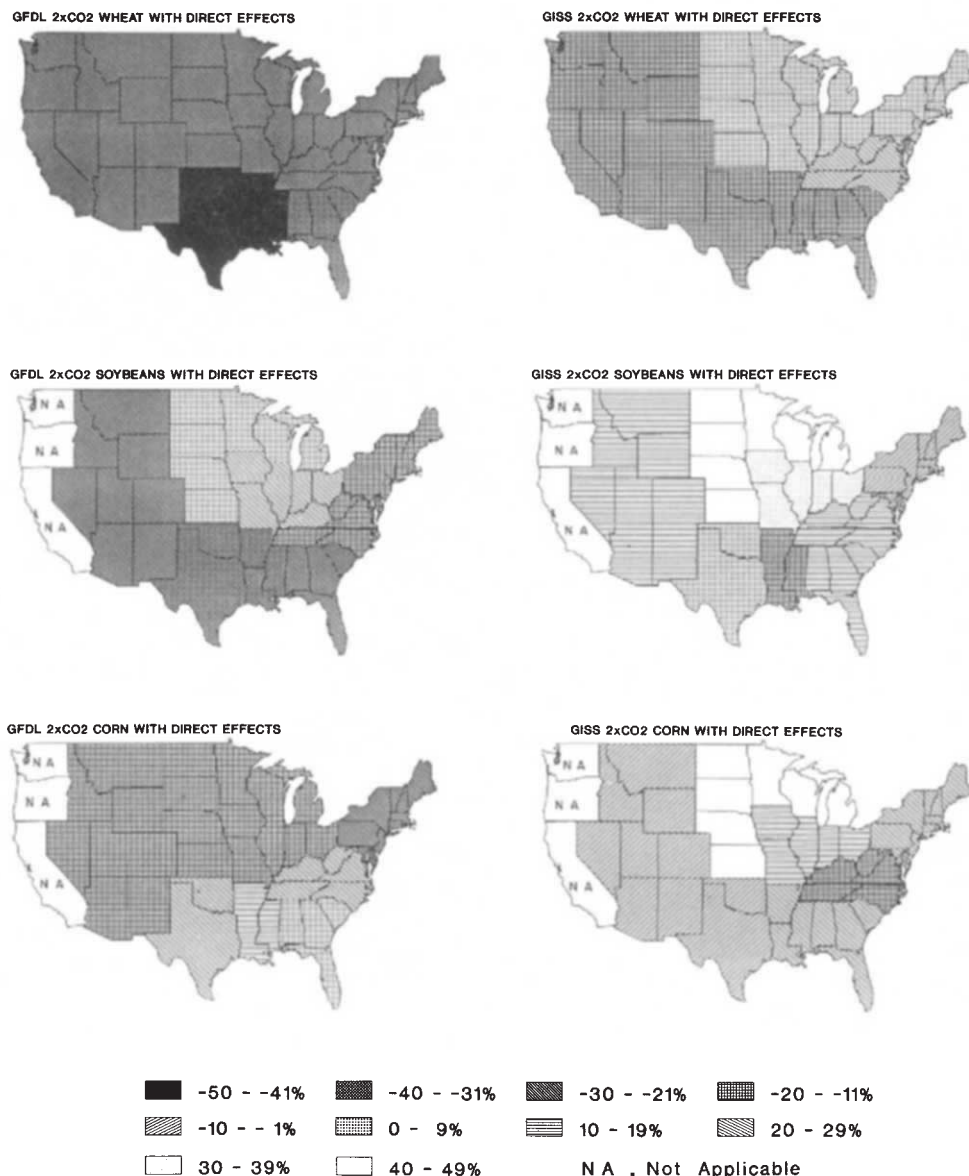


FIG. 1 Percentage change in rain-fed crop yields simulated by the SOYGR0, CERES-Maize, and CERES-Wheat models modified for the direct effects of CO₂ on photosynthesis and transpiration using GISS and GFDL doubled-CO₂ climate-change models. Soybeans and corn were modelled at sites in the following states: Alabama, Arkansas, Florida, Georgia, Kentucky, Louisiana, Mississippi, North Carolina, South Carolina, Tennessee, Virginia, Minnesota, Iowa, Missouri, Wisconsin, Illinois, Indiana, Michigan, Ohio, New York, Pennsylvania; winter wheat and corn were modelled in Nebraska, Kansas, Oklahoma and Texas. Results were extrapolated to other sites on the basis of similarity of current-climate growing conditions.

TABLE 4 Estimated economic consequences of climate change on US agriculture

Model/analysis	Effect			Change from base		
	Con- sumers	Pro- ducers	Total	Con- sumers	Pro- ducers	Total
	(thousand million 1982 \$)					
Base (1981–83 climate, demand and technology)	77.32	17.80	95.12	—	—	—
GISS with doubled CO ₂	86.62	19.39	106.01	+9.30	+1.59	+10.89
GFDL with doubled CO ₂	63.44	21.35	84.78	–13.89	+3.55	–10.33

production activity requires information on yields and usages of inputs or other commodities. This information is from the 1982 United States Department of Agriculture (USDA) Farm Enterprise Data System (FEDS) budgets, the USDA survey of irrigated acreage, Extension budgets, and Soil Conservation Service (SCS) budget sets.

Production is simulated in 64 geographical subregions distinguished by resource endowments. These areas are then grouped into ten larger regions for purposes of land, labour and water supply. The water resource is of particular interest here and is disaggregated into surface and pumped ground water sources. Surface water is available for a constant price within each region, but pumped ground water is provided according to a supply schedule where increasing amounts of water are available for higher prices.

The production and consumption sectors are assumed to be made up of a large number of individuals operating under competitive market conditions. This leads to a model that maximizes the difference between the areas under the demand and supply curves. This area can be interpreted as a measure of economic welfare (ordinary consumers' plus producers' surplus) equivalent to the annual net income (in 1982 dollars) lost or gained by agricultural producers and consumers (both domestic and foreign) as a consequence of climate change. The model is solved as a quadratic programming problem. A more detailed description of the model is found in Cheng and McCarl²⁷.

Economic model simulations

Adjustments in crop yields, irrigated-crop water requirements and irrigation water availability were made in the economic model according to each GCM forecast (Fig. 1 and Table 3). In addition to the modelled crops (maize, wheat, soybeans), the yields for cotton, barley, grain sorghum, rice and alfalfa were adjusted (by the average yield changes of the modelled crops) based on similarity of current-climate growing conditions. Inclusion of these other major crops is needed to account for potential substitution of crops within and across regions and to reflect the role of such crops in livestock production. A model solution is then generated to see how climate change and elevated CO₂ might alter the economics of the agricultural sector relative to a hypothetical 'no climate change' case.

Three solutions were generated with the economic model. These include the yield and water manifestations of each climate-model forecast and a base solution of the 1981–83 economic model. The direction and magnitude of the changes in income, crop acreage and other modelled characteristics between the base and the climate-change solutions indicate potential economic and resource implications (in quantitative terms) of each climate scenario.

Results from the economic model

The economic consequences of climate change are dependent on the GCM projections. This can be seen in Table 4 where

annual aggregate effects on consumers and producers range from a net gain of over \$10,000 million (GISS) to a loss of over \$10,000 million (in 1982 dollars) for GFDL. These estimates are ~8% of the 1982 market value of US crop and livestock production. The GFDL losses are driven by the yield losses shown in Fig. 1 and the substantial increases in irrigation water requirements associated with adverse climate change. (Without the mitigating effect of CO₂ on crop yields, economic losses under GFDL would increase threefold²⁶.) Note that the change in producers' income is positive under GFDL because of prices rising by more than the climate-induced reduction in total production (that is, inelastic demand). Producers in some regions do lose, however, as regional production declines by more than the increase in prices.

Under the GFDL climate-change model, crop prices increase from reduced production of some crops. These price increases work against consumers, whose losses are greater than the gains to producers reported in Table 4. Aggregate price and quantity adjustments for crops and livestock commodities, expressed as indices (weighted by the quantities of each crop), are reported in Table 5. Price changes are slight to moderate across the climate-change projections. GISS yield (increases) and water forecasts result in price declines of 17 and 16% for eight field crops and four livestock commodities, respectively. These price declines are the result of 9 and 6% increases in agricultural output for each group. For GFDL yield and water changes, prices increase by 34 and 8% for the same set of commodities, due to production reductions of 20 and 2% for crops and livestock.

For perspective, we compare these aggregate economic consequences of climate change and elevated CO₂ to the economic effects of other environmental stresses. The economic consequences of anthropogenic tropospheric ozone on US agriculture have been estimated to be \$2,000–\$3,000 million per year in 1982 dollars²⁸ whereas the estimates of a 15% depletion in the stratospheric ozone column amount to ~\$2,500 million²⁹. Thus, the agricultural consequences of climate change under GFDL imply potential economic costs four times greater than these other environmental stresses.

A major policy concern is whether climate change is a food security issue for the United States. The results of these analyses suggest that it is not. For GFDL, the capacity of the US agricultural system to produce food and fibre is reduced, but under GISS production actually increases. US consumers face moderately higher prices under a GFDL climate-change model and the movement of US production into export markets is reduced. Indeed, almost half of the consumers' losses arising under the GFDL climate change accrues to foreign consumers. The analysis here, however, does not consider changes in agricultural production due to climate change in other countries.

These simulations were derived from an economic model based on 1981–83 economic and agronomic conditions. As the full extent of the climate changes projected here will not occur for decades, the sensitivity of the economic estimates were tested

TABLE 5 Agricultural commodity price and quantity indices* for climate-change models (base=1.00)

Climate model	Field crops†		Livestock commodities‡	
	Price	Quantity	Price	Quantity
GISS with doubled CO ₂	0.83	1.09	0.84	1.06
GFDL with doubled CO ₂	1.34	0.80	1.08	0.98

* Indices reported here are Fisher indices, where $P = [(p^1 \cdot q^1/p^0 \cdot q^0)(p^1 \cdot q^0/p^0 \cdot q^1)]^{1/2}$, $Q = [(p^1 \cdot q^1/p^1 \cdot q^0)(p^0 \cdot q^1/p^0 \cdot q^0)]^{1/2}$, and where $p^0(q^0)$ and $p^1(q^1)$ are vectors of prices (quantities) in the original and final equilibria, respectively³.

† Field crops include maize, wheat, soybean, sorghum, cotton, oats, hay (alfalfa and grass hays) and silage.

‡ Livestock commodities include poultry, pork, beef and milk products.

TABLE 6 Effects of climate change on irrigated and rain-fed crop acreage

Region*	Base acreage			GISS, change from base			GFDL, change from base		
	Irrigated	Rain-fed	Total	Irrigated	Rain-fed	Total	Irrigated	Rain-fed	Total
Corn Belt	NA	95.46	95.46	NA	+1.53	+1.53	NA	-4.93	-4.93
Lake States	NA	33.79	33.79	NA	+0.14	+0.14	NA	+3.40	+3.40
Southeast	1.72	10.79	12.51	-0.06	-3.78	-3.84	-0.16	-4.52	-4.68
Delta	3.11	16.77	19.88	+1.08	-11.68	-10.60	+5.91	-9.09	-3.18
Northern Plains	10.28	91.40	101.68	+3.94	-1.12	+2.82	+4.03	-2.14	+1.89
Southern Plains	5.31	49.4	54.70	+1.50	-12.40	-10.90	-0.60	-1.90	-2.50
Mountain	16.14	5.54	21.68	-2.69	+1.46	-1.23	+0.02	-0.27	-0.25
Pacific	7.73	1.94	9.67	-0.21	+1.03	+0.82	+0.26	+0.95	+1.21
Other (Northeast and Appalachian)	NA	19.54	19.54	NA	-5.68	-15.68	NA	-8.76	-8.76
	44.29	324.63	368.92	+3.56	-40.50	-36.94	+9.46	-27.26	-17.80

* Corn Belt region includes the states Illinois, Indiana, Iowa, Ohio and Missouri; Lake States include Michigan, Minnesota and Wisconsin; other regions defined in Table 2. NA, not applicable.

by changing demand and technology parameters in the economic model. Specifically, US domestic and export demands were increased to reflect increases in US and world population, and yields were increased based on historical per annum yield changes, projected until 2050. Increases in domestic and foreign demand increased economic losses for the GFDL climate model. Conversely, technology (through increased yields) offsets the climate-induced losses reported in Table 4 for GFDL if yields increase at rates comparable to the last three decades. Although such yield increases offset the effects of adverse climate, climate change still imposes economic costs (in the form of foregone higher yields in the absence of climate change).

Effects on regional production, irrigated acreage and the environment. Climate change leads to a slight reduction in total US cropped acreage. Aggregate (and regional) acreage adjustments are presented in Table 6. Under GISS, aggregate acreage is reduced due to increased yields. For GFDL, acreage is reduced owing to shifts in production between regions and associated changes in resource availability. Both analyses show reductions in aggregate rainfed acreages. Included are some potentially large regional adjustments, driven by the regional variations in climate change. For example, north or northwest shifts in acreage and production occur for some commodities (Table 6). This implies increased input demands in areas of expanded crop acreage, such as the Pacific and Lake State regions, and corresponding reductions in regions experiencing acreage declines, such as the southeast and Southern Plains. Shifts in regional production patterns imply changes in the status of natural resources. For example, expansion of agricultural production in some areas increases the potential for soil erosion, ground- and surface-water pollution, and loss of wildlife habitats.

Changes in precipitation and temperatures under doubled- CO_2 conditions favour irrigated crop production. Also, rising commodity prices from reductions in total output under the GFDL climate-change model enhance the feasibility of irrigation activities, particularly those associated with groundwater use. As a result, modelled irrigated crop acreage increases in most regions, as seen in Table 6. In the aggregate, the increases vary from 3.5 (GISS) to ~10.0 (GFDL) million acres. Regionally, major increases in irrigated acreage occur in the Northern Plains and in the Delta. The southeast is the only region that shows declines in irrigated acreage, due to the large increase in crop water-use forecast by the crop simulation models. The sustainability of any increase in irrigated acreage was not included in the analysis.

Discussion

Our results show a range of possible outcomes for US agriculture, depending on the severity of climate change and the compensating effects of CO_2 on crop yields. Changes in temperature and precipitation as forecast by the GCMs lead to reductions in yield and increases in crop water demands. As modelled here,

increased atmospheric CO_2 enhances crop yields, mitigating some or all of the climate-induced yield changes. Under the most adverse case of climate change (GFDL), domestic and foreign consumers face moderate increases in real prices. These same price increases benefit US producers.

Some general implications for the US agricultural sector can be drawn. First, because of possible changes in domestic and foreign production under a GFDL climate, the role of the United States in agricultural export markets may change. Second, patterns of agriculture in the United States are likely to shift as a result of changes in regional crop yields and in crop irrigation requirements. Third, concern for future agricultural impacts on important natural resources, especially land and water, seems to be justified. Simulated irrigated acreage increases in most major irrigated regions under the models of climate change examined. In addition, irrigation may increase in more humid regions, a trend that is already occurring.

These results are a preliminary assessment of the potential effects of climate change on agriculture. Critical uncertainties remain, as does the question of appropriate action in light of uncertain but potentially important economic effects. We suggest three sets of considerations to pursue. First, policy makers need to assess whether the probability of the projected agricultural outcomes, coupled with the non-agricultural effects of climate change (for example, sea level rise, damage to natural ecosystems, and increased energy demand) warrant action now to plan for and to reduce the magnitude of climate change. Second, further research must be stimulated to understand likely crop yield, crop water demand and water supply as well as economic adjustments to climate change, thereby reducing the uncertainty of yield projections and other critical inputs to policy analyses. Better projections of changes in regional climate variables, particularly those affecting hydrological balances, are essential. Knowledge of potential change in global agricultural supply and demand is also crucial. Finally, it seems desirable that, for those regions (or countries) that may be adversely affected by climate change, research priorities be set on adapting varieties, species and production techniques to increased temperature and drought stress. □

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Nuclear receptor that identifies a novel retinoic acid response pathway

David J. Mangelsdorf, Estelita S. Ong, Jacqueline A. Dyck* & Ronald M. Evans

Howard Hughes Medical Institute, The Salk Institute for Biological Studies, La Jolla, California 92138-9216, USA

* Physiology and Pharmacology Program, University of California, San Diego School of Medicine, San Diego, California 92093-0636, USA

Molecular cloning and transcriptional activation studies have revealed a new protein similar to the steroid hormone receptors and which responds specifically to vitamin A metabolites. This protein is substantially different in primary structure and ligand specificity from the products of the previously described retinoic acid receptor gene family. By indicating the existence of an additional pathway through which retinoic acid may exert its effects, these data lead to a re-evaluation of retinoid physiology.

THE retinoids comprise a group of compounds including retinoic acid, retinol (vitamin A), and a series of natural and synthetic derivatives that exert profound effects on development and differentiation in a wide variety of systems¹⁻⁸. Retinoic acid has also been shown to induce the transcription of several genes⁹, supporting the hypothesis that it functions in a fashion analogous to steroid and thyroid hormones (reviewed in refs 10, 11). Recently, a new member of the nuclear receptor superfamily was identified as a retinoic-acid dependent transcription factor, referred to as RAR α (refs 12, 13). Subsequently, additional RAR-related genes have been isolated and at least three different RAR subtypes (α , β and γ) are now known in mice and humans¹²⁻¹⁷. These retinoic acid receptors (RARs) share homology with the superfamily of steroid hormone and thyroid hormone receptors and have been shown to regulate specific gene expression by a similar ligand-dependent mechanism¹⁸. The ligand-binding domains of these receptors are highly conserved (>75% amino acid identity), suggesting that they all arose from a common ancestral retinoic acid receptor. Nonetheless, these RAR subtypes are expressed in distinct patterns throughout development¹⁹⁻²¹ and in the mature organism^{13,15-17,22}, indicating that they may mediate different functions. One important question is whether all the actions of retinoic acid are mediated through these proteins or whether additional retinoic acid substrates and regulatory networks exist.

Here we describe the molecular cloning and characterization of a gene for a novel or 'orphan' receptor and report that it

encodes a 462-amino-acid polypeptide that functions as a transcription factor responsive to retinoic acid. Surprisingly, this retinoic acid receptor-like protein is not part of the previously described RAR-subfamily of receptors. These data demonstrate the existence of an evolutionarily parallel regulatory system through which retinoids may exert their control of transcription.

Orphan receptor

Sequential low-stringency screening of a human liver and kidney complementary DNA library with a cDNA fragment encoding the human (h)RAR α DNA-binding domain led to the isolation of several cDNA clones encoding a novel nuclear receptor (referred to as hRXR α). The restriction map and nucleotide sequence of one of these clones, λ XR3-1, are shown in Fig. 1. The λ XR3-1 sequence contains a long open reading frame of at least 462 amino acids ending at the termination codon at nucleotide position 1,462-1,464. The presumptive initiation methionine is placed at the first in-frame ATG at nucleotide position 76-78, although an upstream termination codon is not present. A second potential initiator methionine occurs 27 amino acids downstream. The sequence surrounding both ATG sites conforms well with the consensus described by Kozak²³ for a translation initiation site. *In vitro* translation of RNA derived from the insert (data not shown) shows that λ XR3-1 produces a protein of relative molecular mass 54,000 (M_r , 54 K), close to the predicted M_r of 50,811.

The amino-acid sequence of hRXR α has been compared with that of other members of the steroid hormone receptor superfamily (Fig. 2a). The highest degree of similarity between hRXR α and the other receptors is found in a cysteine-rich sequence of 66 amino acids beginning at hRXR α residue 135 (Fig. 1b). We have shown previously that this region of the human glucocorticoid receptor (hGR), thyroid hormone receptor (hTR) and retinoic acid receptor (hRAR) is the DNA-binding domain^{11,18,24,25}. hRXR α shares striking similarity with the *Drosophila* receptor dXR2C8 (A. Oro, M. McKeown and R.E., manuscript in preparation), both in its DNA-binding (86%) and putative ligand binding (44%) domains. Although the RXR α DNA-binding domain is similar to that of other receptors such as RAR α (61%) and TR β (53%), the putative ligand-binding domain is much less similar (<27% identity) and provides no hint of the nature of the RXR α ligand. A comparison between

transfected with the reporter plasmid ADH-TREp-CAT. Cotransfected Schneider cells were then treated with a series of potential ligand mixes and cell extracts were monitored for CAT activity (Fig. 3). Unexpectedly, mix C, which contained retinol and retinoic acid, elicited a dramatic (>100-fold) induction of CAT activity (Fig. 3a). Other mixes, which included all the known steroid hormones, 1,25-dihydroxyvitamin D₃, thyroid hormone and hydroxycholesterol, did not induce CAT activity. hRXR α also failed to transactivate a reporter without TREp or one containing the glucocorticoid response element (data not shown). These experiments show that RXR α is a retinoid-activated and sequence-specific transcription factor. Because a subfamily of retinoic acid receptors has already been described and the hRXR α gene product does not belong to that subfamily (Fig. 2b), we decided to test various retinoid metabolites for their ability to activate the expressed RXR α protein (see Fig. 3b). At 10⁻⁶ M, retinoic acid elicited the strongest response, followed by retinal and to a much lesser extent, retinyl acetate. There was no induction when cells were exposed to either retinol or retinyl palmitate. As shown in Fig. 3c, retinoic acid produces a saturable dose response curve with a 50% maximal response of 5 $\times$ 10⁻⁸ M.

These results reveal the existence of an alternative molecular pathway of gene regulation mediated by retinoic acid. To

examine the apparent ligand specificities of the RXR and RAR receptor pathways, co-transfection assays were carried out in mammalian cells (Fig. 4). In CV-1 cells hRXR α and hRAR α were expressed constitutively under the control of the Rous sarcoma virus promoter and tested for transactivation using the well characterized reporter plasmid, Δ MTV-TREp-CAT (ref. 18). When transfected cells were challenged with natural vitamin A metabolites, hRXR α and hRAR α showed similar but not identical responses. At 10⁻⁶ M, both proteins responded equally to retinoic acid. But although hRAR α showed a characteristic rank order of potency (retinoic acid > retinal > retinylacetate > retinol > retinyl palmitate = 0) consistent with previous observations¹³, hRXR α responded best to retinoic acid and, to a lesser extent, retinal but not retinylacetate, retinol, or retinyl palmitate (Fig. 4a) as was also the case in *Drosophila* cells (Fig. 3b).

As these results implicate a potential difference in ligand specificity between the RXR and RAR receptor systems, we tested a series of synthetic retinoid-like compounds (Fig. 4b). Synthetic retinoids are often chemically divergent from retinoic acid, but retain potent biological activity^{30,31}. When CV-1 cells transfected with hRXR α and hRAR α were treated with the synthetic retinoids, hRAR α responded maximally to several of the retinoid analogues whereas hRXR α responded only poorly

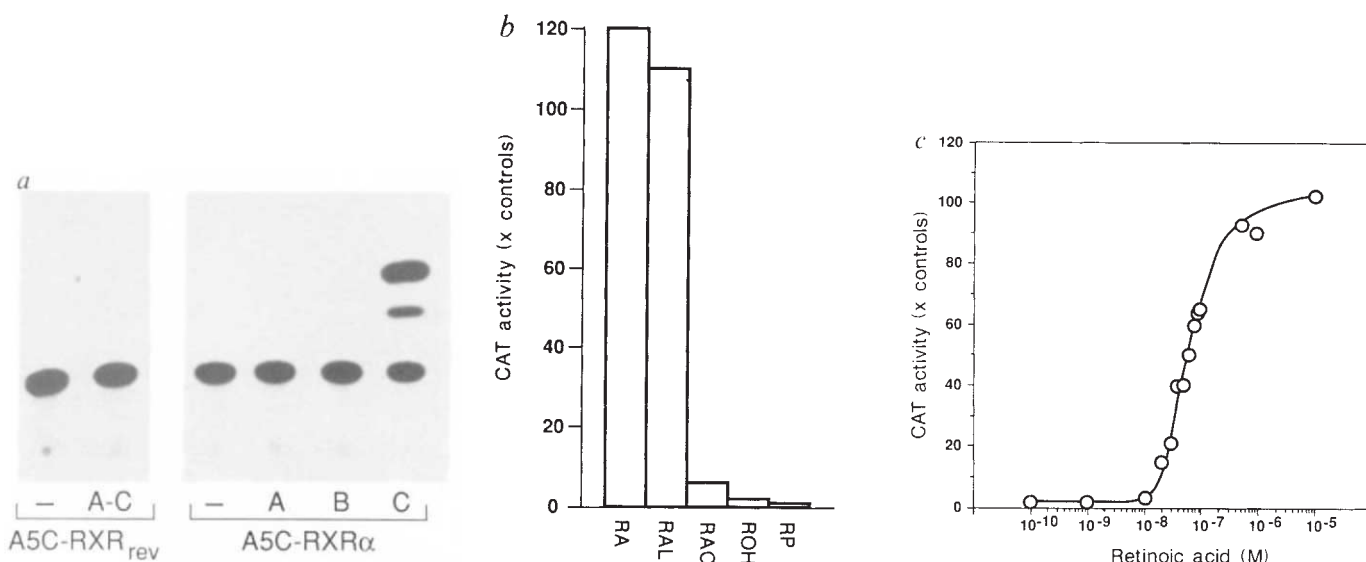


FIG. 3 Retinoic acid induces A5C-RXR α -dependent transcription of the ADH-TREp-CAT reporter in *Drosophila* Schneider cells. *a*, Retinoids specifically induce hRXR α transactivation of CAT. The reporter ADH-TREp-CAT was co-transfected into *Drosophila* Schneider cells with the expression plasmid A5C-RXR_{rev} or A5C-RXR α . The control plasmid A5C-RXR_{rev} contains the reverse orientation of XR3-1 (Fig. 1), and A5C-RXR α contains the full coding region of XR3-1. After transfection, cells were treated with ethanol vehicle (—) or a series of candidate ligand mixtures denoted as A, B, or C. A–C were added as a single mixture to the A5C-RXR_{rev} control, or separately to cells with A5C-RXR α . Mix A included the sterol and steroid hormones pregnenolone (10⁻⁷ M), 17 β -oestradiol (10⁻⁷ M), dihydrotestosterone (10⁻⁷ M), 25-hydroxycholesterol (0.5 μ g ml⁻¹), 1,25-dihydroxyvitamin D₃ (10⁻⁷ M), and dexamethasone (10⁻⁷ M); B included thyroid hormone (10⁻⁷ M), and C included the retinoids retinol (10⁻⁶ M) and retinoic acid (10⁻⁶ M). Only C showed strong RXR-dependent induction. There was no RXR-dependent activation with any candidate ligand on the control reporter ADH-CAT (data not shown). *b*, Natural retinoids induce hRXR α transactivation. Schneider cells were co-transfected with A5C-RXR α and ADH-TREp-CAT and then treated with 10⁻⁶ M retinoic acid (RA), retinal (RAL), retinyl acetate (RAC), retinol (ROH), retinyl palmitate (RP). Transactivation is expressed as induction of CAT activity (x control levels) in retinoid-treated cells compared with ethanol-treated control cells. *c*, hRXR α dose response to retinoic acid. Schneider cells were co-transfected with A5C-RXR α and ADH-TREp-CAT and then incubated with increasing amounts of retinoic acid. CAT activity is expressed as amount of induction (x controls) in treated versus untreated

control cells and represents the average of two experiments.

METHODS. *Drosophila melanogaster* Schneider line 2 (S2) cells⁴³ were seeded at 2 $\times$ 10⁶ per 35 mm² culture dish and maintained in Schneider medium (GIBCO) supplemented with penicillin, streptomycin and 12% heat-inactivated fetal bovine serum (Irving Scientific). Cells were transiently co-transfected with 10 μ g plasmid DNA by calcium phosphate precipitation⁴⁴. Effector constructs (4.5 μ g per dish) A5C-RXR α and control A5C-RXR_{rev} are constitutively expressed under the control of the *Drosophila* actin 5C promoter²⁹. The reporter plasmid ADH-TREp-CAT (0.5 μ g per dish) contains a palindromic thyroid response element^{25,45} inserted into position -33 (with respect to the transcription start site) of a pD33-ADH-CAT background⁴⁴. This reporter contains the distal promoter of the *Drosophila* alcohol dehydrogenase gene linked to the bacterial CAT gene. A reference plasmid (0.5 μ g per dish) containing the β -galactosidase transcription unit driven by the actin 5C promoter (M. Koelle and D. Hogness, unpublished) and pGEM4 (Promega) (4.5 μ g per dish) were included in transfections. Candidate ligands were dissolved in dimethylsulphoxide and/or ethanol and diluted in ethanol for delivery to cells (0.1% v/v of solvent in media). All experiments involving retinoids were conducted in subdued light. Candidate ligand preparations were added 24 h after transfection and cultures were maintained in the dark until collected 60 h after transfection. Cell lysates were centrifuged and the supernatant assayed for β -galactosidase⁴⁶ and units per microlitres of activity were calculated. CAT assays (normalized to β -galactosidase activity) were incubated for 75 unit-hours as described⁴⁷, typically 150 units for 30 min.

if at all (Fig. 4b). Two of the synthetic retinoids, SRI-8520-30 and TTNPB (Ro 13-7410), fully activated hRAR α at concentrations similar to retinoic acid. In contrast, these analogues only partially activated hRXR α , with a maximal effect an order of magnitude less than retinoic acid.

In a comparison of dose responses in CV-1 cells, Fig. 4c shows that hRAR α has a higher sensitivity to retinoic acid than hRXR α , whose response has not yet saturated at 10^{-5} M. The experiments shown in Figs 3 and 4 provide strong evidence that the ligand for hRXR α is either retinoic acid itself or a closely related metabolite or structural analogue.

Tissue distribution

To determine the tissue distribution of hRXR α gene expression, poly(A⁺) RNAs isolated from a variety of adult rat tissues were size-fractionated, transferred to a nylon filter, and hybridized with the hRXR α cDNA. The distribution of RXR α messenger RNA in the rat reveals a pattern of expression (Fig. 5) that is distinct from the retinoic acid receptors which fail to show pronounced expression in visceral tissue^{13,15,17,22}. The rat RXR α mRNA seems to be a single species of 4.8 kb which is expressed in many tissues, but most abundantly in the liver, muscle, lung, kidney and heart.

An RXR gene family

To determine whether there is an RXR gene family, we carried out a series of Southern blot analyses. High-stringency hybridiz-

ation of a labelled DNA fragment derived from λ XR3-1 to human DNA digested with restriction endonucleases produced a similar number of bands in every digestion, consistent with a single genetic locus (Fig. 6, left panel). When the hybridization conditions were relaxed, however, many additional bands were observed in the products of each (Fig. 6, right panel). Inspection of this hybridization pattern revealed that it was unrelated to similar analyses described for hRAR α (ref. 13). These observations indicate the presence of at least one other locus related to the RXR gene in the human genome. Consistent with these results, we are now analysing an additional human orphan receptor gene which, on the basis of sequence comparison, is similar to hRXR α and is referred to as hRXR β (data not shown). The existence of an RXR gene family is supported by the report from Hamada *et al.*³² of a murine cDNA which appears to be homologous to hRXR β , but whose function is unknown. The presence of the related *Drosophila* receptor, XR2C8 (Fig. 2a), together with genomic Southern analyses from several other animal species using hRXR α as a probe (data not shown) indicates that the RXR gene family has been conserved throughout the animal kingdom.

Discussion

A second pathway for retinoid action. This report provides evidence for a retinoid response pathway that is evolutionarily distinct from that mediated by the previously described retinoic acid receptor subfamily. The observation that retinoic acid is

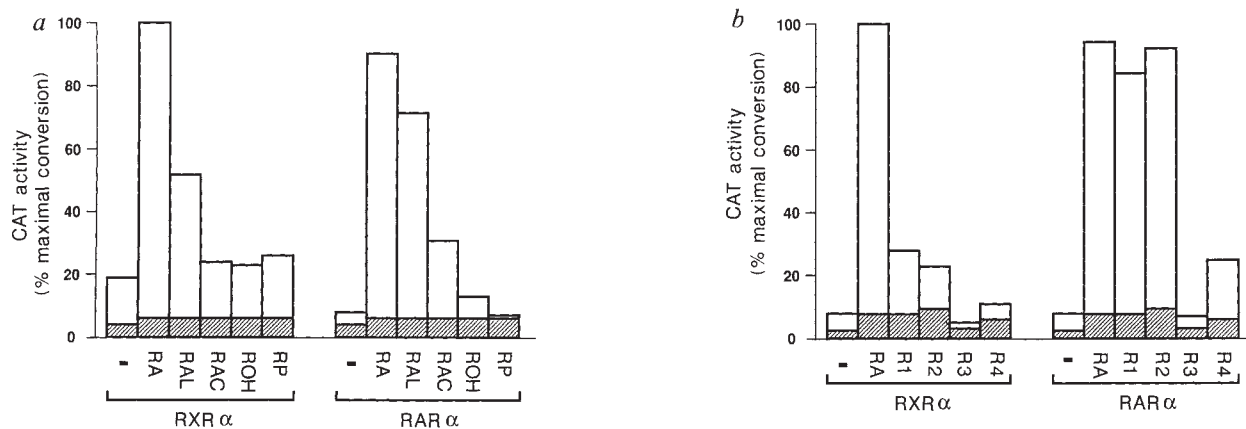
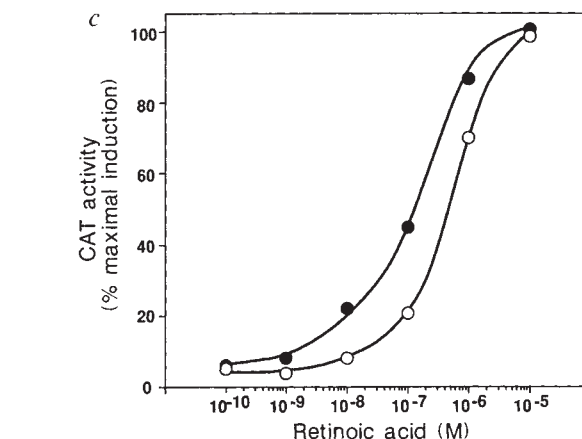


FIG. 4 Comparison of hRXR α and hRAR α transactivation in mammalian CV-1 cells. *a*, Natural retinoid metabolites induce both hRXR α and hRAR α transactivation. CV-1 cells were co-transfected with the reporter Δ MTV-TRE_p-CAT and either control plasmid, RS-hRXR α or RS-hRAR α . Cells were then exposed to the various retinoid metabolites as shown in Fig. 3b at 10^{-6} M. No CAT activity was seen with any receptor in combination with the control reporter Δ MTV-CAT (data not shown). The levels of induced CAT activity for RS-hRXR α and RS-hRAR α (open bars) or control plasmid (hatched bars), are superimposed and plotted as percentages of the maximal response observed in this experiment. *b*, Synthetic retinoids differentially induce hRXR α and hRAR α transactivation. CV-1 cells were co-transfected as in *a* and then treated with the following synthetic retinoids: R1, SRI-8520-30 (4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-4-iodo-2-anthracenyl)-benzoic acid; SRI International); R2, TTNPB or R₀13-7410 (ethyl *P*-(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propenyl]-benzoic acid; Hoffman La-Roche); R3, etretinate or R₀10-9359 (ethyl all-*trans*-9-(4-methoxy-2,3,6-trimethyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate; Hoffman La-Roche); R4, etretin or R₀10-1670/054 (ethyl all-*trans*-9-(4-methoxy-2,3,6-trimethyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid; Hoffman La-Roche). The final concentration of retinoids was 10^{-6} M. The levels of CAT activity are plotted as in *a*. *c*, hRXR α (○) and hRAR α (●) dose response to retinoic acid. CV-1 cells were co-transfected with RS-hRXR α and RS-hRAR α as in *a* and then incubated with increasing amounts of retinoic acid. CAT activity is expressed as per cent maximal induction ($\times$ controls) in treated versus untreated control cells and represents the mean of three experiments. **METHODS.** Construction of the mammalian expression vector RS-hRAR α



has been described¹³ and a similar protocol was used to introduce the λ XR3-1 insert into pRS and create RS-hRXR α . Construction of the reporter plasmid Δ MTV-TRE_p-CAT has also been described²⁵. CV-1 cell culture, co-transfections, and CAT assays were as outlined¹⁸. Retinoids were delivered to cells as described in Fig. 3, 36 h before collecting cells for CAT assays.

the most potent inducer of hRXR α transactivation suggests that it may be the natural RXR ligand, although another metabolite or structural analogue cannot be excluded. To date we have been unable to demonstrate high-affinity binding of [3 H]retinoic acid to hRXR α extracted from cells transfected with the RXR expression plasmid. Such binding is confounded, in part, because of the high levels of cellular retinoic acid-binding protein (ref. 33). This problem has also hindered the generation of convincing binding data with the RARs. High-affinity retinoic acid binding to RAR β and γ has yet to be shown, although specific binding has been reported with RAR α (refs 12, 13). In the case of RXR, one explanation may be that retinoic acid is not the highest-affinity ligand. In cultured mammalian cells retinoic acid is converted rapidly to more polar, hydroxylated metabolites ($t_{1/2}$ = 3.5 h in F9 cells)^{34,35}. It is possible that one of these metabolites is a higher-affinity ligand for hRXR α . According to this hypothesis, retinoic acid could serve as a precursor that is metabolized to a more active form. Such a metabolic pathway would be reminiscent of vitamin D action, in which it was originally postulated that 25-hydroxy-vitamin D₃ was the active principle. Not until the discovery of a more polar metabolite specifically bound to chromatin³⁶ was 1,25-dihydroxy-vitamin D₃ identified as the true hormone. Another example is the production of the female sex steroid, oestrogen,

through the aromatization of the male sex steroid, testosterone. This example is particularly interesting because although these two hormones are closely related, the ligand-binding domains of their respective receptors share only marginal (25%) homology³⁷. By this analogy, it is not unreasonable to presume that RAR and RXR recognize chemically similar or identical molecules, even though their ligand-binding domains are only distantly related.

Re-thinking retinoid physiology. Unlike the RARs, which reveal only limited expression in the adult, RXR α is highly expressed in the liver, which is a major site of vitamin A storage, metabolism and mobilization. Although its functions are not yet known, RXR α is a candidate regulator of vitamin A metabolism itself. It is also found in the kidney and heart, and to a lesser extent, in various other tissues, predicting a potential role in adult physiology. The similarity of the DNA-binding domain of RXR α to that of the RARs suggests they might recognize common regulatory sequences. Indeed, as established in this report, both RXR and RAR can activate transcription through the palindromic thyroid hormone response element. Thus, RXR α is likely to regulate a partially overlapping set of RAR-responsive genes and, therefore, would be candidate for mediating some of the developmental effects of retinoic acid. In this context, it will be particularly important to determine whether the RXR family is

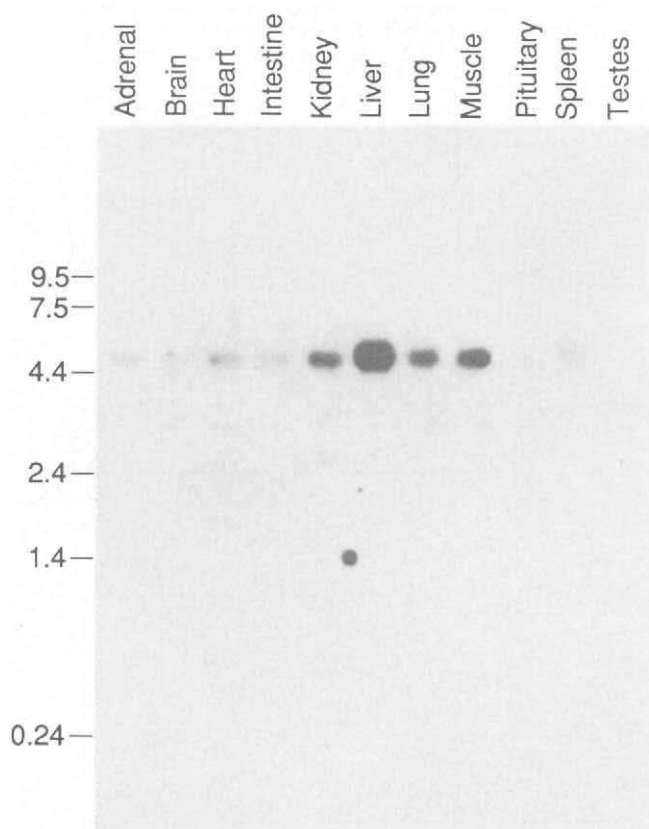


FIG. 5 Northern blot analysis of hRXR α mRNA in rat tissues.

METHODS. Total RNA was prepared from adult male rat tissues⁴⁸ and poly(A⁺)-selected by oligo(dT)-cellulose chromatography. Poly(A⁺) RNA (10 μ g) was separated by electrophoresis in a 1% agarose gel containing formaldehyde, transferred to a Nytran filter (Schleicher and Schuell)⁴⁹, and hybridized under stringent conditions with the 1.9-kb λ XR3-1 EcoR1 insert described in Fig. 1. Hybridization was at 42 °C in a buffer containing 50% formamide, 5 $\times$ Denhardt's solution, 5 $\times$ SSPE, 0.1% SDS, 100 μ g ml⁻¹ salmon sperm DNA, 200 μ g ml⁻¹ yeast RNA, and 32 P-labelled probe. The filter was then washed twice with 2 $\times$ SSC, 0.1% SDS at 22 °C and twice at 50 °C. Autoradiography was for 24 h at -70 °C with an intensifying screen. The RNA ladder size markers (in kb; BRL) are aligned at the left of the autoradiograph.

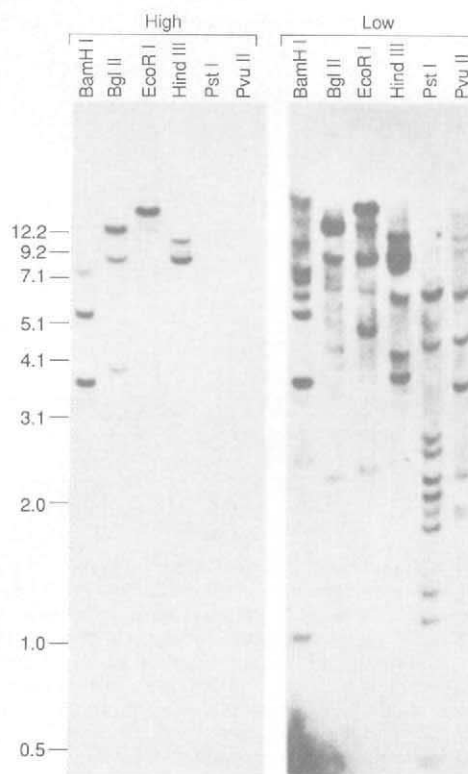


FIG. 6 Human genomic DNA Southern blot analyses of hRXR α . Two human placenta genomic DNA Southern blots were prepared in parallel with identical DNA samples. The blots were hybridized at high or low stringency with a $\sim$ 1,200-bp 32 P-labelled fragment of λ XR3-1 that included the coding portions of the DNA and ligand-binding domains (λ XR3-1 sequence 459-1,631). **METHODS.** Human placenta DNA was restricted with the indicated enzymes, separated in a 0.8% agarose gel (10 μ g per lane) and transferred to nitrocellulose⁵⁰ and hybridized at 42 °C in the low-stringency buffer described in Fig. 1 or at high stringency in identical buffer containing 50% formamide. The low-stringency blot was washed twice at room temperature and twice at 50 °C in 2 $\times$ SSC, 0.1% SDS. The high-stringency blot was washed twice at room temperature in 2 $\times$ SSC, 0.1% SDS and twice at 65 °C in 0.5 $\times$ SSC, 0.1% SDS. Autoradiography was for 24 h at -70 °C with an intensifying screen. The DNA ladder size markers (in kb; BRL) are aligned at the left of the autoradiograph.

expressed in the developing embryo and to determine its relationship to the already known RAR system. The discovery in RXR of a second transduction pathway with distinct pharmacological properties may lead to a better understanding of

how retinoids can elicit an enormous diversity of biological responses and suggests that retinoid metabolism may provide important clues to the identification of new ligands. □

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Molecular genetic basis of the histo-blood group ABO system

Fumi-ichiro Yamamoto, Henrik Clausen, Thayer White, John Marken & Sen-itiroh Hakomori

The Biomembrane Institute and University of Washington, 201 Elliott Avenue W., Seattle, Washington 98119, USA

The histo-blood group ABO, the major human alloantigen system, involves three carbohydrate antigens (ABH). A, B and AB individuals express glycosyltransferase activities converting the H antigen into A or B antigens, whereas O(H) individuals lack such activity. Here we present a molecular basis for the ABO genotypes. The A and B genes differ in a few single-base substitutions, changing four amino-acid residues that may cause differences in A and B transferase specificity. A critical single-base deletion was found in the O gene, which results in an entirely different, inactive protein incapable of modifying the H antigen.

THE blood group ABO antigens, originally identified by Landsteiner on erythrocytes, were the first major alloantigens recognized in humans¹, and are responsible for failure of unmatched blood transfusions. The ABO antigens are found on other cell types (mainly epithelial), and changes in these antigens associated with development and oncogenesis have been the subject of renewed interest^{2,3}. In fact, phylogenetic and ontogenetic studies have established that the ABO antigens evolved earlier on epithelial cells than on blood cells^{2,4}, and therefore should be considered as histo-blood group antigens². Chemical charac-

terization of the determinants that define the histo-blood group ABO(H) specificities showed that these antigens were oligosaccharides with the minimal determinant structures shown in Fig. 1 (refs 5-7). In the late 1960s, the biosynthetic pathway of these determinants was established, and it was found that A individuals express $\alpha 1 \rightarrow 3$ N-acetylgalactosaminyltransferase (A transferase) activity whereas B individuals express $\alpha 1 \rightarrow 3$ galactosyltransferase (B transferase) activity⁸⁻¹¹. AB individuals express both activities and O individuals express neither.

Although the A and B antigens were shown to be inherited as simple Mendelian dominant characters¹², and the three-allelic model for the inheritance of the ABO genes was proposed by Bernstein¹³ early in this century, the molecular genetic mechanism controlling expression of ABO antigens has remained unknown. The A and B genes are thought to encode the glycosyltransferases to synthesize either A or B antigen, whereas the O gene is thought to be silent or give rise to an enzymatically inactive protein⁷. This model is supported by the observation that A and B transferases have overlapping substrate specificities^{14,15}, and by the immunological cross-reactivity of anti-A transferase antibodies with B transferases¹⁶⁻¹⁸. The status of the O gene and its potential protein product is unclear. The expression of an immunologically related protein in O individuals was reported¹⁷, but this has not been confirmed¹⁹. Our recent studies with monoclonal antibodies to the A transferase indicated that no immunologically related protein is generated from the O gene¹⁸. Recently, the soluble form of A transferase was isolated from human lung tissue²⁰ and from

human intestinal mucosa²¹. Based on partial amino-acid sequence, we have cloned and sequenced complementary DNA encoding this transferase²².

Here, we describe cloning and sequencing of complementary DNA from cell lines of different ABO status, using the A-transferase probe, in order to determine the sequence differences between ABO genes. We also confirmed the structural ABO differences by analysis of genomic DNAs prepared from 14 blood samples with different ABO status as evidenced by digestibility with diagnostic restriction enzymes.

Cloning and sequencing of allelic cDNAs

A total of four cDNA libraries were constructed in λ gt10 with poly(A)⁺ RNA from cell lines of different histo-blood group ABO status. Human colon adenocarcinoma cell lines SW948, SW48, COLO205, and SW1417 were chosen. These cell lines were derived from patients of blood type O, AB, unknown, and B, respectively, and express similar messages to that of A transferase as determined by northern hybridization²².

As shown in Table 1, the two clones FY-59-5 and FY-59-7 (from the MKN45 cDNA library) were identified as representing A-gene alleles because they showed identical sequences for corresponding regions, and the deduced amino-acid sequences of these clones matched that of purified A transferase. However, they showed different 5' and 3' ends, as well as different splicing patterns, although the significance of this is unclear. Similar premature messages have been reported for other cloned glycosyltransferases^{23,24}. Four cDNA clones (FY-65-1, FY-65-10, FY-65-15, FY-65-18) obtained from the cDNA library of SW948 (phenotype O, genotype OO) showed identical nucleotide sequences, and were judged as representing an O gene allele. Either this cell line is homozygous, or only one of the two alleles has been cloned. cDNA clones from SW48, (the AB cell line), were divided into two groups: clones FY-66-1, FY-66-2, FY-66-3, FY-66-7 belong to the same group, whereas clone FY-66-9 differs by several base substitutions (Fig. 2), resulting in four amino-acid substitutions (Fig. 3). On the basis of nucleotide sequence similarities between FY-66-1, FY-59-5, and FY-59-7, we assumed that the group represented by FY-66-1 is the A allele and the other represented by FY-66-9 is the B allele at the ABO locus.

Nucleotide sequences of cDNA clones from the SW948 (phenotype O, genotype OO) library and these clones were compared. cDNA clones were identical to the presumed A allele (FY-66-1) except for a single-base deletion. This deletion, located close to the N terminus, results in a shift of the reading frame (Fig. 3). As previously known glycosyltransferases have the catalytic domain close to the C terminus²⁵, this shift presumably leads to translation of an enzymatically inactive protein. To extend these findings, two additional cDNA libraries (FY-68

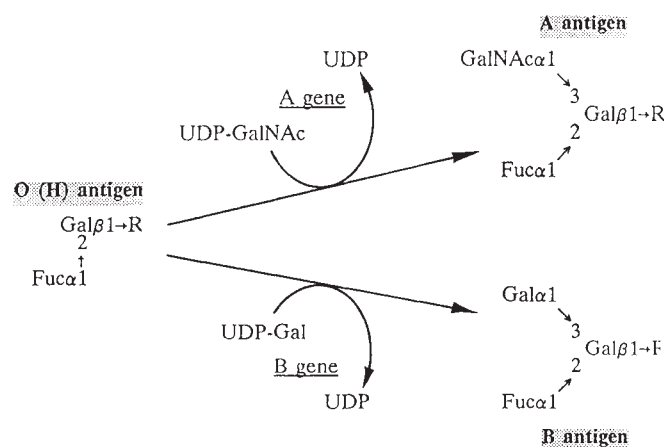


FIG. 1 Biochemical relationship between A, B and H antigens.

TABLE 1 Classification of DNA clones

Source of RNA	Pheno-type	Blood type	cDNA clones	Possible genotype
MKN-45	A	ND	59-5, 59-7	A
SW948	O	O	65-1, 65-10, 65-15, 65-18	O
SW48	AB	AB	66-1, 66-2, 66-3, 66-7	A
			66-9	B
COLO-205	O	ND	68-6, 68-11, 68-12, 68-14, 68-15	O
SW1417	B	B	69-2, 69-7	O
			69-3, 69-4, 69-8	B

Each cDNA clone was classified by the library. Allelic cDNAs were separated based on nucleotide sequence. Phenotype of the cell line, blood type of host, and possible genotype are indicated. ND, not determined.

and -69) were constructed, and cDNA clones were obtained and sequenced. All the cDNA clones (FY-68-6, -11, -12, -14 and -15) from COLO205 showed the identical nucleotide sequence; hence again we do not know whether both alleles have been cloned. SW1417 cDNA clones were divided into two groups as for SW48. Clones FY-69-2 and FY-69-7 comprise one group and clones FY-69-3, -4 and -8 the other. The latter group possessed a nucleotide sequence identical to FY-66-9 (B allele), whereas the group represented by FY-69-7 showed a sequence identical to cDNA clones from O cell line SW948 (O allele).

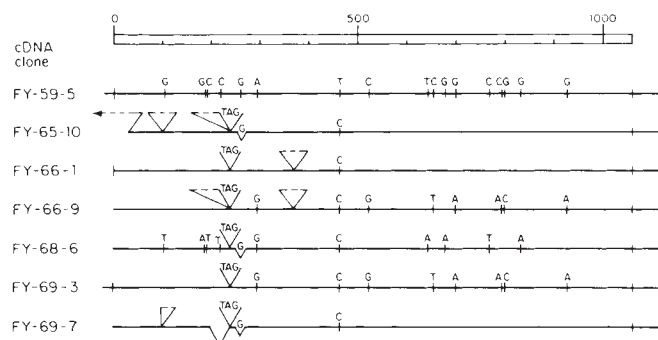


FIG. 2 Nucleotide sequence comparison of clones from five cell lines of different ABO status. FY-59-5 was compared with representative allelic cDNA clones from various cell origins. Insertions are shown above the line, deletions below the line. The nucleotide sequences in various clones were identical to FY-59-5, except those indicated above the line. We identified seven single base substitutions between predicted A and B clones (Table 1). Compared to the nucleotide sequence of A transferase cDNA clone FY-59-5 (ref. 22), these are A, C, C, G, C, G, G in A allele and G, G, T, A, A, C, A in B allele (positions 294, 523, 654, 700, 793, 800 and 927 respectively). One consistent single base deletion (G at position 258) was present in the predicted O allelic clones.

METHODS. cDNA libraries were constructed in λ gt10 with poly(A)⁺ RNA from cell lines. Random hexamer was used for priming the reverse transcription, as in the MKN45 cDNA library construction²². About one million recombinant phage plaques from each library were screened with a ³²P random primed-probe from FY-59-5 insert, which had the longest coding sequence among A transferase cDNAs from MKN45 cell line. Positive clones were plaque purified after the tertiary screening. DNA was prepared and subcloned into the EcoRI site of plasmid pT7T3 (Pharmacia). Each clone was restriction mapped and characterized by the 5' and 3' ends and the presence or absence of introns. Nucleotide sequencing of representative clones was performed with single-stranded DNA templates produced by superinfection with helper phage M13K07. Several oligodeoxynucleotides complementary to FY-59-5 sequences were used as primers, as well as an M13 universal primer for Sanger method sequencing³⁶. The accumulated nucleotide sequence data were aligned for individual cDNA clones and compared with each other and data from FY-59-5 insert. The splicing pattern of individual clones varied significantly, and therefore that of FY-59-5 was used as a standard.

TABLE 2 Genotyping of genomic DNA from blood samples at ABO locus

Specimen no.	Blood phenotype	Status at			Possible genotype
		Position 1 (nucleotide 258)	Position 2 (nucleotide 523)	Position 3 (nucleotide 700)	
1	A	—/—	A/A	A/A	AA
2	A	O/—	A/A	A/A	AO
3	A	O/—	A/A	A/A	AO
4	B	O/—	A/B	A/B	BO
5	A	O/—	A/A	A/A	AO
6	O	O/O	A/A	A/A	OO
7	O	O/O	A/A	A/A	OO
8	O	O/O	A/A	A/A	OO
9	O	O/O	A/A	A/A	OO
10	AB	—/—	A/B	A/B	AB
11	B	O/—	A/B	A/B	BO
12	B	O/—	A/B	A/B	BO
13	B	O/—	A/B	A/B	BO
14	AB	—/—	A/B	A/B	AB

The dash (—) indicates the non-O (*Bst*EII-cleavable, *Kpn*I-uncleavable) allele at this position. O/—, a combination of *Kpn*I-cleavable O allele and non-O allele. A, *Bss*HII cleavable allele at position 2, or *Hpa*II cleavable allele at position 3. B, *Nar*I-cleavable allele at position 2, or *Alu*I-cleavable allele at position 3. A/A and A/B, combination of these alleles at each position.

DNA from buffy coat fraction of blood samples with clearly defined ABO phenotypes were analysed as described (Fig. 4 legend). Status is represented by the diagnostic restriction enzyme cleavage site specific for each allele. Status at position 1 was determined for the presence of single base deletion by Southern blot analysis after *Bst*EII or *Kpn*I digestion. Status at positions 2 and 3 was determined by PCR and restriction enzyme digestions (*Nar*I/*Bss*HII and *Alu*I/*Hpa*II for positions 2 and 3 respectively). Possible genotype was inferred from status at these sites, and from phenotype.

The genotype of SW1417 is therefore BO. We found the same single-base deletion in cDNAs from COLO205 (phenotype O) as in SW948, in addition to several base substitutions.

Genetic basis of ABO polymorphism

Characterization of the various cDNA clones identified possible diagnostic nucleotide differences responsible for the ABO polymorphism. Four consistent nucleotide substitutions leading to amino-acid changes (residues 176, 235, 266 and 268; see Fig. 3) were found between predicted A and B allelic cDNAs. All the predicted O allelic cDNA clones shared a single nucleotide deletion in the coding region (nucleotide position 258; see Fig. 2), indicating that the lack of transferase activity in O individuals is due to a shift in the reading frame.

To verify the observed sequence differences for the ABO polymorphism, genomic DNA from 14 blood samples (buffy coat fraction) with known ABO phenotype, as well as these cell lines, were studied. Nucleotide sequence analysis of cDNA clones identified allele-specific restriction enzyme cleavage sites at three of the four substitutions between A and B allelic cDNAs, as well as the single-base deletion found in O allelic cDNAs (Fig. 4a).

We took advantage of these restriction sites for preliminary genotyping by the polymerase chain reaction (PCR)²⁶. Because of the lack of information on genomic DNA sequence at the ABO locus, we searched for working pairs of oligodeoxynucleotides. Two pairs of primers (fy-43 and -31, and fy-47 and -29) were satisfactory and used for PCR amplification of fragments (467 and 621 base pairs respectively), which cover the four important base substitutions between A and B genes. The cleavage sites susceptible to diagnostic restriction enzymes were detected by the presence of fragments in 12% polyacrylamide gels, stained by ethidium bromide to detect the first (Fig. 4b and second (Fig. 4c) differences between A and B alleles. The *Nar*I fragments of 205 and 262 base pairs (bp) were obtained with DNA from SW48 (lane 3) and SW1417 (lane 5, Fig. 4b). The 203- and 264-bp fragments were obtained after digestion with *Bss*HII of DNA from all the cell lines examined, but the 467-bp fragment remained for SW48 (lane 8) and SW1417 (lane 10, Fig. 4b), indicating heterozygosity of these cells for *Bss*HII (A allele) and *Nar*I (B allele) at this position. The other cell lines, MKN45 (lane 6), SW948 (lane 7) and COLO205 (lane 9) were homozygous for *Bss*HII (A allele). Similarly, as shown in Fig. 4c, SW48 and SW1417 were found to be heterozygous for

*Hpa*II (A allele; lanes 8 and 10) and *Alu*I (B allele; lanes 3 and 5). The other cell lines were homozygous at this second site for *Hpa*II. These results confirmed the presence of these nucleotide differences in genomic DNA as well as cDNA.

The single base deletion was detected by Southern blot analysis²⁷ (Fig. 4d). Restriction enzyme digestion with *Bst*EII (lanes 1–5) and *Kpn*I (lanes 6–10) of genomic DNA from the five cell lines followed by Southern transfer and hybridization with FY-59-5 insert probe clearly confirmed our finding of single base deletion in genomic DNA. In addition, we found homozygosity for this deletion in two O cell lines. The MKN 45 cell line (lanes 1 and 6) was homozygous for the *Bst*EII site, or without deletion. SW948 (lanes 2 and 7) and COLO 205 (lanes 4 and 9) were homozygous for the *Kpn*I site. This again supports our theory of O allele inactivation by single base deletion. The SW1417 cell line (lanes 5 and 10) was heterozygous, as predicted.

We also analysed genomic DNAs from blood samples (buffy coat) of different ABO phenotype. As shown in Table 2, all four O samples had the single base deletion (at position 1) in both alleles. All the A and B samples showed at least one functional allele, devoid of the single base deletion. The AB samples showed two functional alleles. All the B and AB samples so far tested showed the presence of *Nar*I and *Alu*I sites (at position 2 and 3 respectively). Consequently, the data provide strong evidence in favour of the proposed genetic basis of the ABO polymorphism.

Primary product of the O gene

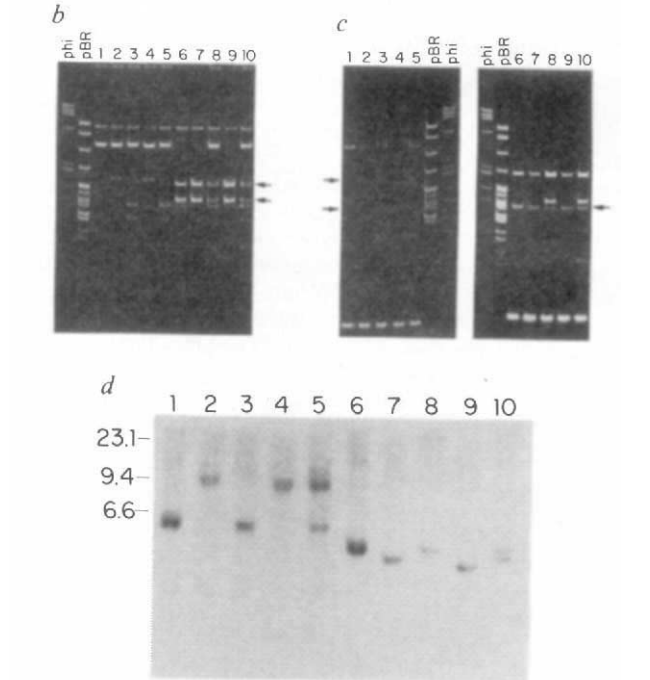
Histo-blood group phenotype O cells express a message similar to that of A and B alleles²². It is now clear that the corresponding DNA sequence is identical to the A allele, except for a single-base deletion (258G, Fig. 4a) in the coding region close to the N terminus of the protein. Such a deletion shifts the reading frame, resulting in translation of an entirely different protein. It is therefore unlikely that O individuals express a protein immunologically related to the A and B transferases, which agrees with the absence of the cross-reacting protein in O cells with specific monoclonal antibody directed to a soluble A transferase¹⁸.

Discussion

Our results clearly establish the molecular basis for ABO polymorphism. The A and B genes directly encode the A and B glycosyltransferases, and the difference in their specificities is

	210	220	230	240	250
FY-59-A	SEVDYIVCVD	VDMEFRDHVG	VEILTPLFGT	LHPGFYSSR	EAFYERRPQ
FY-65-O					
FY-66-A	*****	*****	*****	*****	*****
FY-66-B	*****	*****	*****	*****S*****	*****
FY-68-O					
FY-69-B	*****	*****	*****	*****S*****	*****
FY-69-O					
	260	270	280	290	300
FY-59-A	SQAYIPKDEG	DFYIIGGFFG	GSVQEVQRLT	RACHQAMMVD	QANGIEAVWH
FY-65-O					
FY-66-A	*****	*****	*****	*****	*****
FY-66-B	*****	*****M**A**	*****	*****	*****
FY-68-O					
FY-69-B	*****	*****M**A**	*****	*****	*****
FY-69-O					
	310	320	330	340	350
FY-59-A	DESHLNKYLL	RHKPTKVLS	EYLWDQQLG	WPAVLRLKLF	TAVPKNHQAV
FY-65-O					
FY-66-A	*****	*****	*****	*****	*****
FY-66-B	*****	*****	*****	*****	*****
FY-68-O					
FY-69-B	*****	*****	*****	*****	*****
FY-69-O					
	360				
FY-59-A	RNP-				
FY-65-O					
FY-66-A	****				
FY-66-B	****				
FY-68-O					
FY-69-B	****				
FY-69-O					

residues (Cys and Arg) in other alleles. Asterisks indicate residues identical to FY-59-A. Different residues are shown in bold type. Question marks indicate the unidentified sequence due to absence of corresponding nucleotide sequence in cDNAs. The symbol (—) denotes the stop codon.



35 cycles of reaction (denaturation at 94 °C for 90 s, annealing at 50 °C for 2 min, and incubation at 72 °C for 3 min with 5 s extension for each cycle). The samples were extracted with phenol–(chloroform: isoamyl alcohol, 24:1 v/v) ethanol-precipitated and resuspended in 20 µl of 1 mM Tris (pH 7.5), 1 mM EDTA. Next, 5 µl of the DNA was digested with restriction enzymes and subjected to 12% PAGE. The gels were stained with ethidium bromide and photographed. Southern hybridization was performed with 10 µg of DNA. DNA was electrophoresed through 1% agarose gel after digestion with *Bst*EII (lanes 1–5) or *Kpn*I (lanes 6–10) and transferred onto Nylon membrane (Amersham). The filter was baked and prehybridized in 50% formamide, 5× SSPE, 5× Denhardt's, and 0.1% SDS solution at 42 °C (2 h) and then hybridized overnight at 42 °C with a [³²P] random prime-radiolabelled probe from the FY-59-5 insert. The filter was washed in 2× SSC, 0.1% SDS at room temperature three times and then 1× SSC, 0.1% SDS at 68 °C (1 h). Final wash was in 0.1× SSC, 0.1% SDS at 68 °C. DNA markers are phi-X/*Hae*III (phi) and pBR 322/*Msp*I (pBR).

METHODS. Genomic DNAs were prepared by proteinase K-SDS method²⁷. PCR reaction was performed with 1 µg of DNA with Taq DNA polymerase with a DNA Thermal Cycler (Perkin Elmer-Cetus). The synthetic oligodeoxynucleotides used are: fy-29, 5'-CGTTCGTCTGCTAAACCAAG; fy-31, 5'-GAAATCGCCCTGTCCTT; fy-43, 5'-GGATCCAGGGGTGCACGGCCGGCGGC; fy-47, 5'-TGCTGGAGGTGCGCGCCTAC. fy-43 and fy-31 were used for amplification in (b) and (c) and fy-47 in (c). The *Bam*HI site in fy-43 is artificial. There were

based on four amino-acid substitutions (residues 176, 235, 266 and 268). Transfection of O cells with reconstructed DNAs encoding the crucial sequences for A and B specificities resulted in conspicuous expression of A and B antigens respectively (manuscript in preparation). The O gene does not produce such functional transferases because of a structural rather than expressional failure.

Additional polymorphism within the ABO system is based on subgroups⁷. The A1-A2 subgroup is based on expression of A transferases with different kinetic properties²⁸, leading to both quantitative and qualitative differences in the A carbohydrate antigens²⁹. It is predicted that a few amino-acid substitutions beyond the ones found distinguishing the A and B genotypes could be responsible for the subgroup polymorphism. Further studies on this possibility are in progress.

The present results show that the nucleotide sequences of ABO alleles are closely related, which has implications for the direction of evolution of this gene. O alleles from SW948 and SW1417 have a single base deletion as compared with the A allele from SW48. The O allele from COLO205 has the same single base deletion at the same site as in SW948 and SW1417, but this allele also has several base substitutions. It is possible that additional mutations accumulated in O alleles, because the

gene became non-functional through the shift of the encoding frame. A and B transferase activities have been observed in tissues from various species including pigs³⁰ and rats³¹. However, the presence of the same three conservative single-base substitutions in two B alleles from SW48 and SW1417 implies the relatively recent divergence of A and B alleles.

Recently the H gene has been isolated³², but as yet the genetic basis of the polymorphism involved in these histo-blood group systems has not been established. However, it seems likely that similar mechanisms underlie the polymorphism induced by these genes.

Expression of the histo-blood group ABH antigens undergoes marked changes during differentiation and oncogenic transformation^{2,3}. In stratified squamous epithelia, A and B cell-surface antigens are acquired at a defined stage of differentiation, and deleted or reduced in malignant or premalignant cells^{2,3}. On the other hand, O individuals may express 'incompatible A antigens' in adenocarcinoma cells^{33,34}. This may be linked to the long-standing observation that the incidence of adenocarcinomas of most organs in O individuals is low in comparison to that in A individuals³⁵. Therefore, the molecular genetic mechanism controlling ABO antigen expression during differentiation and oncogenesis is of paramount interest. □

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LETTERS TO NATURE

Gamma-ray bursts from young supernova remnants

Serge Pineault*

Institute of Astronomy, University of Cambridge,
Madingley Road, Cambridge CB3 0HA, UK

COMETS falling onto neutron stars have been suggested¹⁻⁴ as a cause of γ -ray bursts. In the original form of the idea^{1,2}, it was assumed that the neutron star remained at the centre of the Oort cloud of comets belonging to its progenitor, so that bursts would be infrequent. Here it is noted that a neutron star may well acquire a large velocity at birth, causing it to drift through the Oort cloud. As it crossed the most densely populated regions, γ -ray bursts would occur frequently. Young supernova remnants may therefore be the site of some of the observed bursts, particularly the less energetic, more rapidly recurring type known as

soft- γ -ray repeaters⁵. SN1987A may become a source of γ -ray bursts in a matter of decades.

The detection of circumstellar material around nearby stars⁶ suggests (but does not require) that Oort-type⁷ comet clouds may exist around other stars. One might worry that the combined effects of radiation and ablation in the more rigorous environment (extreme ultraviolet fluxes, stellar winds) prevailing around the more massive stars ($\geq 6-8 M_{\odot}$) needed to form a neutron star might make the formation and survival of comets somewhat more problematical⁸. But a number of other factors must be taken into account. During the protostellar phase, for example, the wind outflow geometry may be as crucial as the strength of the wind itself. Indeed strongly anisotropic flows, such as the bipolar outflows observed from some young stellar objects⁹, may have a minimal impact on cometary bodies forming in a protoplanetary disk. Likewise ultraviolet photons will have a short path length in a dense disk. One should also note that the actual strength of both radiative fluxes and winds is a steep function of stellar mass. By way of comparison, the mass loss rate¹⁰ for an $8-M_{\odot}$ main-sequence star ($\sim B4$) is only $10^{-9} M_{\odot} \text{ yr}^{-1}$ as compared to 6×10^{-8} and $2 \times 10^{-7} M_{\odot} \text{ yr}^{-1}$ for stars of 16 and $25 M_{\odot}$ respectively (or $\sim 10^{-6} M_{\odot} \text{ yr}^{-1}$ for O stars). This suggests

* Permanent address: Département de physique, Université Laval, Ste-Foy, Québec, Canada, G1K 7P4.

that a significant number of comets may actually be able to survive (at least around stars less massive than about $10 M_{\odot}$). Finally if a substantial fraction of cloud comets have been acquired by capture (see ref. 11 for a review), their survival is obviously independent of early stellar evolution. Bearing the above remarks in mind, I adopt the working hypothesis that Oort-type clouds are likely to be present around at least some (and possibly a significant fraction) of neutron-star progenitors.

Recent models¹²⁻¹³ of the Solar System comet cloud show that the number density $n_c(r)$ of comets at distance r from the Sun can be expressed as $n_c(r) = k_n r^{-n-2}$ where k_n is determined from observations. For lack of better information, I use the same parameterization with $n = 2.5$ (refs 12,13) and assume a comet population proportional to the total primordial mass $M_0 \equiv m_0 M_{\odot}$ of the system, from which⁴ ($r_{AU} \equiv r/(1 \text{ AU})$)

$$n_c(r) \approx 2.0 \times 10^{17} m_0 r_{AU}^{-9/2} \text{ AU}^{-3} \quad (1)$$

The total cloud mass is then $M_c \approx 2.7 \times 10^{-5} m_0 (r_-/10^3 \text{ AU})^{-1.5} (\bar{M}_c/10^{15} \text{ g}) M_{\odot}$ where $\bar{M}_c$ is the mean cometary mass, r_- is the inner boundary of the cloud which may be as close as 10^3 AU and the cloud has been assumed spherical.

The calculation of the impact rate $\dot{N}$ follows the method previously described for the case of a random encounter between a neutron star and other comet-bearing stars^{3,4}, that is, $\dot{N} = \pi p^2 n_c v$, where $p^2 = 2GM_{ns} \mathcal{R}_{ns}/v^2$ is the capture impact parameter and v is the velocity with which the neutron star of mass $M_{ns} \equiv m_{ns} M_{\odot}$ is assumed to be moving through the cloud. The effective radius of the neutron star, $\mathcal{R}_{ns} \approx 3.5 \times 10^7 m_{ns}^{-7/27} \text{ cm}$, exceeds the physical radius R_{ns} because of the drag introduced by the magnetic field¹⁴ (note that the above expression for $\mathcal{R}_{ns}$ is five times that used in ref. 4; this accounts for the 'indirect' capture of comets²). The magnetic field B has been eliminated by assuming a flux-conserving family of neutron stars for which $BR^2 = \text{constant}$ ^{3,4} (normalized to 10^{12} G for a $1-M_{\odot}$ star). Note that because $\mathcal{R}_{ns} \propto B^{4/9}$ these impact rates for a newly formed neutron star may be an underestimate because these stars may have a substantially higher field at birth. The impact rate is thus

$$\dot{N} \approx 2.2 m_0 (m_{ns}/1.4)^{20/27} v_{10}^{-1} r_3^{-9/2} \text{ yr}^{-1} \quad (2)$$

where $v_{10} \equiv v/10 \text{ km s}^{-1}$ and $r_3 \equiv r/10^3 \text{ AU}$. The total number N_t of comets accreted by the neutron star by the time it has emerged from the cloud is $N_t = \int \dot{N} dt \approx 260 m_0 (m_{ns}/1.4)^{20/27} v_{10}^{-2} r_3^{-7/2}$ (where r_3 is r_- in units of 10^3 AU).

Because of the steep radial dependence of $n_c(r)$, most impacts occur near the inner edge of the cloud. If T_f is the time during which the accretion rate exceeds f times its maximum value, then for a purely radial trajectory³ $T_f = 500 v_{10}^{-1} r_3^{-3} (f^{-1/(n+2)} - 1) \text{ yr}$, which for $f = 0.1$ and $n = 2.5$ gives $T_{0.1} \approx 330 v_{10}^{-1} r_3 \text{ yr}$. The inner edge of the cloud would be reached after a time of the order of $500 v_{10}^{-1} r_3 \text{ yr}$ after the supernova explosion.

The observed fluence S of a burst is related to the mass of the comet by

$$M_c \approx 5.4 \times 10^{17} (m_{ns}/1.4)^{-4/3} D^2 S \eta^{-1} \text{ g} \quad (3)$$

where D is the source distance in parsecs, η is the fraction of released energy emitted as γ -rays and an approximate mass-radius relation of the form $MR^3 = \text{constant}$, normalized at 10 km for a $1-M_{\odot}$ neutron star, has been used for simplicity^{3,4}.

The velocity of the neutron star depends on the details of the supernova explosion. The large observed proper motions of pulsars¹⁵ indicate that these are formed by asymmetric explosions that impart a substantial velocity to the neutron star. In a recent study, Narayan and Ostriker¹⁶ found evidence for two distinct pulsar populations having velocities of 50 km s^{-1} and 150 km s^{-1} respectively. If pulsars are representative of the overall galactic neutron-star population, these are then the velocities to use in estimating the burst rate $\dot{N}$.

Even for a symmetric supernova explosion it is, however,

TABLE 1 Pre-supernova and post-supernova parameters for disrupted systems

Case	a_0	m_2^0	m_1^0	v_0	$v_{ns,\infty}$
A	3	5	10	67	22
B	30	5	10	21	7.1
C	9	8	16	49	16
D	30	8	16	27	8.9

Supernova explosion assumed symmetric and pre-supernova orbits circular. Masses in $M_{\odot}$, distances in AU, and velocities in km s^{-1} .

possible for a neutron star to break away from its comet cloud if it is part of a binary system. As one cannot rule out the possibility that pulsars do not represent the entire galactic neutron-star population, it is worthwhile to consider this situation as well. For simplicity, I consider the pre-supernova orbits to be circular, the relative velocity of the two stars at separation $a_0 \text{ AU}$ being $v_0 \approx 30 (m_0/a_0)^{1/2} \text{ km s}^{-1}$. The binary is disrupted if the mass loss $\Delta M \equiv M_0 - M_{ns}$ exceeds half the total pre-supernova mass M_0 , or if $m_1 - m_2 > 2m_{ns}$ (ref. 17). (Star 1 is assumed to be the faster-evolving star and all stellar masses denoted by a lower-case m are normalized to the Sun's mass, $m_i \equiv M_i/1 M_{\odot}$).

If $\Delta m \geq m_0/2$, the neutron star escapes to infinity with a velocity with respect to the original centre of mass given by $v_{ns,\infty} = (m_2/m_0) v_0$ (ref. 18). If $\Delta m < m_0/2$, the system remains bound but with new orbital eccentricity $e = \Delta m/(m_2 + m_{ns})$ (ref. 19) and semi-major axis $a/a_0 = (m_{ns} + m_2)/(2m_{ns} + m_2 - m_1^0)$, and furthermore its new centre of mass acquires a velocity v_{ncm} with respect to the original centre of mass given by $v_{ncm}/v_0 = (\Delta m/(m_2 + m_{ns})) (m_2/m_0) = em_2/m_0$. In the case of a symmetric explosion in a binary system, either $v_{ns,\infty}$ or v_{ncm} is thus the relative velocity v to be used in the calculation of the impact rate given by equation (2).

To illustrate the range of parameters expected, a number of idealized systems have been considered, assuming all supernovae to form a $1.4 M_{\odot}$ neutron star. Table 1 shows systems where the masses of the stars differ by a factor of two and for which disruption is certain. For each pair of masses, the representative semi-major axes have been chosen such that the first one is the smallest compatible with no mass transfer taking place during the evolution of the primary²⁰, whereas the largest one corresponds to the 'peak' in the separation distribution of binaries²¹. Note that the ratio $v_{ns,\infty}/v_0$ is independent of the semi-major axis. Table 2 consists of binary systems where spherical mass loss is not sufficient to unbind the binary. Note that cases I to K are based on mass-transfer binary models²²⁻²⁴ proposed for SN1987A. In all three cases the total mass of the system at birth is $22 M_{\odot}$ and the pre-supernova mass of star 1 is the smallest. The 'missing' mass in case J is in a massive common envelope.

From the sample binary systems displayed in the tables, it is clear that relative velocities in the range ~ 5 to $\sim 40 \text{ km s}^{-1}$ are easily produced (and much larger velocities possible as shown by case J) even for spherically symmetric explosions. On the basis of these results and the empirical evidence from pulsar proper motions discussed before, it seems reasonable to consider a velocity of 50 km s^{-1} as 'typical'. Using $m_0 = 22$, and assuming an inner boundary at $r_3 = 1$ (ref. 12), one would thus predict that, some 100 years following a supernova explosion, a γ -ray burst source would appear with an initial burst rate of some 7 bursts per year, falling to less than one per year some 60 years later. By the time the source had died, the neutron star would have accreted some 200 comets. As a comparison, before the supernova explosion, the steady-state impact rate², scaled up to a $20-M_{\odot}$ system, is only expected to be $\sim 4.5 \times 10^{-4} \text{ yr}^{-1}$ (the reason for this low rate is that the loss-cone of the much more numerous comets that have low semi-major axes is not filled). If substantial comet clouds exist around other stars, it is thus likely that a fraction of the observed sources of γ -ray burst are

TABLE 2 Binary parameters for systems which remain bound

Case	a_0	m_2^0	m_1^0	v_0	v_{ncm}	a	e
E	3	10	10	76	30	12	0.75
F	30	10	10	25	9.3	120	0.75
G	9	16	16	57	24	56	0.84
H	30	16	16	31	13	560	0.84
J*	11	18	4	42	4.7	13	0.13
J*	0.04	3.4	5.5	440	140	0.29	0.85
K*	0.46	16	6	210	39	0.63	0.26

Supernova explosion assumed symmetric and pre-supernova orbits circular. Masses in $M_\odot$, distances in AU, and velocities in km s^{-1} .

* Mass transfer models suggested for SN1987A (refs 22–24). The total pre-supernova mass in case J is also $22 M_\odot$, because the two stellar cores are surrounded by a massive common envelope.

associated with young supernova remnants, many of which may be as yet undetected because, for example, they either occur in dense molecular clouds and hence never become radio supernova remnants²⁵, or are simply still too small to be identified. Assuming a bursting lifetime of $\sim 1,000$ yr per source ($f = 0.01$ in equation (3), which is probably a conservative estimate) and a supernova rate of one every 30 years in the Galaxy, there could be up to ~ 30 such sources in the Galaxy, a fraction of which are probably already included in lists of γ -ray burst sources.

These sources are expected to be associated with young stars. Such a separate class has already been proposed⁵ for the three so-called soft- γ -ray repeaters. One source has been tentatively identified with the supernova remnant N49 in the Large Magellanic Cloud^{26,27}. Ignoring the unusually large and atypical burst of 5 March 1979, I note that the observed impact rate ($\sim 10 \text{ yr}^{-1}$ in 1982; ref. 28) can be produced with reasonable values of the parameters in equation (2), although the observed fluences ($S = 10^{-6}$ – $10^{-7} \text{ erg cm}^{-2}$) require large but not impossibly high comet masses ($\sim 10^{20-21} \text{ g}$). The offset of the source position from the centre of the supernova remnant²⁷ remains somewhat of a puzzle if the γ -ray burst source is the neutron star associated with the formation of the supernova remnant.

The other soft- γ -ray repeater (SGR) for which a large number of bursts have been observed^{29,30} is SGR1806–20. Although possible ‘periodicities’ of 2.4 years and 4 months have been proposed, the outbursts seem to occur in clusters having time-scales ranging from an hour to a month, the number of bursts per interval of fluence S varying as $dN/dS \approx S^{-2}$. In my model, intervals of a few months or years are quite typical (see equation (2)) and a distribution of cometary masses naturally produces a corresponding fluence distribution (see equation (3) and refs 2 and 3). The fast recurrence times which are also sometimes observed (for example, 10 events in one hour on 16 November 1983) could also be explained as originating from different fragments of the same initial comet.

Could SN1987A become such a γ -ray burst source, given that comet clouds around stars may be the norm rather than the exception? Whether SN1987A occurred in a binary system or not is not known. It has been argued²³, however, that a binary model may account for several of the anomalous features of SN1987A. Of the three binary models depicted in Table 2, model J is probably the only one still compatible with the present light curve (Ph. Podsiadlowski, personal communication). With a predicted velocity of nearly 150 km s^{-1} for this model, one could expect SN1987A to turn on as a γ -ray burst source within the next 30 years or so, bursting at an initial rate of 2–3 per year. Note that the issue of the binary nature of SN1987A is of little importance here because the above estimate is equally valid (within a factor of three) if the progenitor of SN1987A was a star that underwent an asymmetric explosion of the type suggested by the galactic pulsar data. Although the number of bursts observable from Earth with present detectors would be considerably smaller than this number (at 55 kpc, a burst of $S \sim 10^{-8} \text{ erg cm}^{-2}$, near the present detection limit, would require a

minimum comet mass of $\sim 10^{19} \text{ g}$, much larger than average by Solar System standards³¹), technological advances in the next decades are almost certain to alter completely the outlook for detection of even the weakest bursts. $\square$

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Using comet light-curve asymmetries to predict comet returns

Michel Festou*, Hans Rickman† & Lars Kamélt†

* Observatoire de Besançon, UA 389 du CNRS,
41^{bis} Avenue de L'Observatoire, F-25044 Besançon, France

† Astronomiska Observatoriet, Box 515, S-75120 Uppsala, Sweden

THE gravitational attractions of the Sun and planets do not account completely for the orbital motions of short-period comets, which tend to return to perihelia with some delay or advance as compared with the gravitational solution. Debate over the physical cause of these ‘nongravitational effects’ has continued from the time they were discovered, in the early nineteenth century. Uncertainty over their origin has made prediction of these effects for any particular comet impossible. To clarify the roles of the radial and transverse components of the nongravitational force, and thus to arrive at an accurate picture with predictive power, we have used observational data on gas production rates from comets as a diagnostic of the force. The shapes of the production curves, based mostly on visual light curves, correlate very well with the nongravitational delays or advances of a number of comets, and we have used this correlation to predict a substantial advance of the recent perihelion passage of comet P/Brorsen–Metcalf, as verified by observations.

A perturbing acceleration with radial and transverse components, j_r and j_t , in the orbital plane of the comet will change the orbital period P during one revolution by

$$\Delta P = \frac{6\pi}{n^2 a \sqrt{1-e^2}} \left\{ e \int_0^P j_r \sin f dt + p \int_0^P \frac{j_t}{r} dt \right\} \quad (1)$$

where a and e are the orbital semi-major axis and eccentricity. The mean motion is $n = 2\pi/P$, and the semi-latus rectum is $p = a(1 - e^2)$. The angle f is that formed by the position of the comet, the Sun and the perihelion, and t is time. ΔP is the offset of the time of perihelion passage.

The first term in curly brackets vanishes if $j_r(-f) \equiv j_r(f)$, but Bessel¹ suggested long ago that in the absence of such a symmetry it might dominate. Cometary astrometry was not accurate enough to test this hypothesis. Whipple² focused attention on a rotational thermal lag which he expected to act persistently in the same transverse direction throughout the perihelion passage, and hence suggested the transverse force component as the origin of the observed effects. To calculate cometary orbits, Marsden *et al.*³ introduced the expression

$$(j_r, j_t) = (A_1, A_2) \cdot g(r) \quad (2)$$

where $g(r)$ is a function simulating the variation of H_2O evaporation flux with heliocentric distance r (AU), and A_1 and A_2 are constants to be calculated along with the orbital elements. This model has a symmetric j_r component which gives no contribution to ΔP ; introducing equation (2) into equation (1), we obtain

$$\Delta P = \frac{6\pi\sqrt{1-e^2}}{n^2} A_2 \int_0^P \frac{g(r)}{r} dt \quad (3)$$

Recently, doubts have arisen over the physical validity of equation (2), and arguments have accumulated for ascribing more of the observed ΔP to j_t rather than to j_r . The nongravitational effect in P/Halley is probably dominated by the j_t contribution⁴, and for some comets an improved fit to astrometric positions is obtained by applying a time shift to equation (2) according to the peak of the light curve⁵. We have therefore searched for a quantitative statistical correlation between non-gravitational effects and perihelion asymmetries of gas production curves. Such a correlation is expected if the j_t contribution to ΔP is important, but not otherwise.

To estimate the asymmetries of gas production curves we have used water production rates determined either directly from OH emission measurements obtained with the International Ultraviolet Explorer, or from the empirical law

$$\log Q_{(OH)}(s^{-1}) = 32.0 - 0.4m_H \quad (4)$$

using a correlation⁶ between the OH production rate and the heliocentric magnitude m_H . The latter is calculated from the observed visual magnitude (m_v) corrected for an inverse-square dependence of apparent brightness on geocentric distance. Observed magnitudes were mostly taken from the International

Comet Quarterly archive. Deriving the exact shape of the light curve demands some care: from comparisons of such magnitude sets with direct measurements, and by arguing that visual observers are more prone to underestimate than to overestimate the total brightness of a comet⁷, we discarded the low brightness estimates and took the envelope of all the observations to be the most probable real light curve.

The asymmetry of the gas production curve is then

$$\int_0^P Q_g \sin f dt = e^{-1} \sqrt{\frac{a(1-e^2)}{GM_\odot}} \int_{a(1-e)}^{a(1+e)} (Q_a - Q_b) dr \quad (5)$$

where G is the gravitational constant and $M_\odot$ is the solar mass. We denote by Q_a and Q_b the gas production rate (Q_g) after and before perihelion at any given heliocentric distance, and introduce the asymmetry parameter E which measures the pre- or post-perihelion excess of gas production

$$E = Q_m^{-1} \int_{a(1-e)}^{a(1+e)} (Q_a - Q_b) dr \quad (6)$$

The normalization factor Q_m is arbitrarily chosen to be the maximum water production rate of the comet.

We have assembled 17 short-period comets for which we could derive estimates of E corresponding to time periods where A_2 values are available (ref. 8 with a small addition from the Minor Planet Circulars). These values are either those of single linkages or averages over longer periods where A_2 remained nearly constant. Periods involving strong orbital perturbations are generally avoided. For comets P/Forbes and P/Encke we used different time intervals with different A_2 values, bringing the number of entries to a total of 22. Table 1 lists the comets with their perihelion distances, mean A_2 coefficients for the indicated time intervals, and our estimated values of Q_m and E . Also listed are the nongravitational effects ΔP , calculated from equation (3), and the corrected values $\Delta P'$ to be described below. The estimated probable errors of E are generally very large, reflecting the problems in reliably defining the shapes of the gas production curves, so it is not generally possible to reach conclusions for single objects.

Some care must be taken when direct measurements of $Q_g(t)$ are lacking: if the comet is dust-rich so that the dust coma contributes most of the visual brightness, equation (4) may be misleading. Most of the suspected dust-rich comets, however, have direct measurements that allow the visual-magnitude-based estimates to be rectified (P/Stephan-Oterma) or disposed of altogether (P/Halley). Another problem is that for comets for which A_2 changes rapidly a representative gas production curve

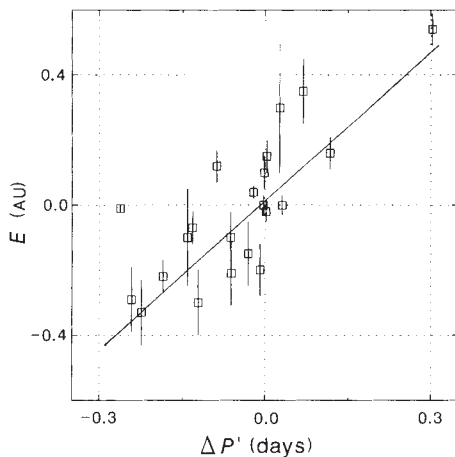


FIG. 1 Using the results in Table 1, we show the correlation between the light-curve asymmetry parameter E and the perturbation of the orbital period, or time offset of perihelion passage $\Delta P'$, corrected for differing semi-major axes. Each table entry is marked by an open square and error bars corresponding to the estimated uncertainty of E .

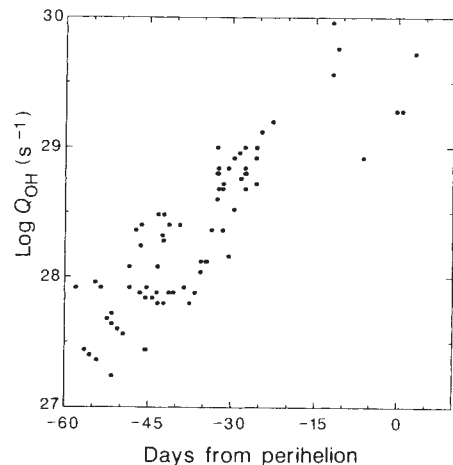


FIG. 2 Based on published estimates of the total visual magnitude of comet P/Borsen-Metcalf 1919 III, and the translation into OH production rate as given by equation (4), we plot the estimated gas production rates of the comet by separate symbols (filled circles). Note the lack of observations of the comet after perihelion passage.

TABLE 1 Comet orbits, nongravitational effects and gas production data

Comet	q (AU)	$10^8 A_2$ (AU d ⁻²)	Time interval	log Q_m	E (AU)	ΔP (d)	$\Delta P'$ (d)
Borrelly	1.45	-0.038	1932-1981	28.85	-0.10 ± 0.08	-0.084	-0.063
Churyumov-Gerasimenko	1.30	0.0125	1969-1982	27.85	0.30 ± 0.20	0.0276	0.0274
Comas Solá	1.77	-0.010	1943-1962	27.55	-0.20 ± 0.08	-0.014	-0.0090
Crommelin	0.74	-0.0003	1873-1984	28.55	0.10 ± 0.05	-0.012	-0.0011
d'Arrest	1.16	0.117	1970-1982	28.65	0.54 ± 0.05	0.291	0.303
Encke a	0.34	-0.0226	1885-1895	29.35	-0.29 ± 0.10	-0.0774	-0.242
b	0.34	-0.0211	1891-1905	29.7	-0.33 ± 0.10	-0.0723	-0.224
c	0.33	-0.0114	1924-1937	29.55	-0.30 ± 0.10	-0.0390	-0.122
d	0.34	-0.0059	1950-1964	29.1	-0.21 ± 0.10	-0.020	-0.062
e	0.34	-0.0030	1970-1985	29.15	-0.15 ± 0.10	-0.010	-0.031
Faye	1.66	0.0069	1910-1948	28.0	-0.02 ± 0.03	0.0022	0.0018
Forbes a	1.54	0.0733	1929-1948	27.5	0.16 ± 0.05	0.114	0.118
b	1.51	-0.0781	1961-1980	27.6	-0.07 ± 0.05	-0.125	-0.132
Giacobini-Zinner	1.00	-0.060	1972-1985	28.9	-0.22 ± 0.05	-0.186	-0.185
Halley	0.59	0.0155	1835-1984	29.9	0.35 ± 0.10	4.10	0.0695
Honda-Mrkos-Pajdušáková	0.56	-0.044	1948-1980	28.45	-0.01 ± 0.01	-0.181	-0.262
Kopff	1.57	-0.091	1970-1983	28.85	-0.10 ± 0.15	-0.137	-0.141
Schaumasse	1.19	-0.039	1911-1960	28.8	0.12 ± 0.05	-0.122	-0.088
Stephan-Oterma	1.57	-0.0030	1867-1981	28.3	0.00 ± 0.03	-0.046	-0.0026
Tempel 2	1.37	0.0016	1961-1983	28.55	0.15 ± 0.05	0.0025	0.0036
Tuttle	1.03	0.013	1912-1980	28.40	0.00 ± 0.03	0.108	0.032
Wolf-Harrington	1.62	-0.0145	1971-1985	27.45	0.04 ± 0.02	-0.0121	-0.021

may be hard to determine. Such is the case for comets P/Comas Solá and P/Wolf-Harrington, and the observational data for these comets may not be representative. On the other hand, we have not seen any example of a comet with both a stable A_2 and a rapidly changing gas production curve.

The table reveals a tendency for ΔP , A_2 and E to have the same sign, suggesting that the desired correlation may exist⁵. ΔP is an ill-suited parameter because the same acceleration A_2 can give rise to widely differing ΔP effects for comets with different mean motions (equation 3). As a plot of A_2 against E still shows a large scatter, we return to equation (1) and express the jet acceleration by

$$(j_r, j_t) = \frac{Q_g m u_g}{M} (\cos \eta, \sin \eta \cos \psi) \quad (7)$$

where M is the mass of the nucleus, u_g is the net outflow speed of the gas, m is the mass of a gas molecule, η is the angle between the net outflow direction and the direction to the Sun, and ψ is an azimuthal angle describing the orientation of the projection of the outgassing vector onto a plane perpendicular to the Sun-comet line with respect to the direction of orbital motion. From thermal model calculations⁹ it seems that u_g does not vary drastically between different orbital positions, and we may also expect that η remains small. Introducing equation (7) into equation (1), we can thus replace these parameters by orbital averages, and using equations (5) and (6), we get

$$\Delta P \approx \frac{6\pi m \langle u_g \rangle}{n^2 \sqrt{aGM_\odot}} \frac{Q_m}{M} \times \{(\cos \eta)E + \sqrt{GM_\odot/p} \sin \eta_e T\} \quad (8)$$

where we have introduced an effective lag angle η_e such that $\sin \eta_e = \langle \sin \eta \cos \psi \rangle$, and put

$$\int_0^P Q_g (1 + e \cos f) dt = Q_m T \quad (9)$$

The first factor in equation (8) varies with the orbital elements as $a^{5/2}$. Let us define a standardized nongravitational effect $\Delta P' = \Delta P (a/3.5 \text{ AU})^{-5/2}$, as listed in Table 1, which the comet would experience with a standard semi-major axis of 3.5 AU, and which measures only the physical trigger of the effect. Equation (8) then shows that a linear relationship between E and $\Delta P'$ is expected if the radial component is indeed the principal agent of the nongravitational effect and if Q_m/M is reasonably constant.

Figure 1 shows a scatter diagram of E as a function of $\Delta P'$, and indeed we see a remarkably good correlation, indicating that the nongravitational effects are strongly influenced by the perihelion asymmetries of the gas production curves. The radial component of the jet force therefore seems to be a principal contributor to the observed effects, and becomes dominant for $|E|$ in the range 0.1–0.2 AU. For a short-period comet with only one or two observed apparitions, it is practically impossible to determine ΔP from an orbital solution, so that ephemerides for the subsequent apparition are uncertain. But using the regression line in Fig. 1, one may apply an estimate of E (if enough observations are available) to predict $\Delta P'$ and hence ΔP . As a test case of practical interest, we took comet P/Brorsen-Metcalf before its recent recovery. This comet had two apparitions in 1847 and 1919 with a period of ~ 70 years, and the upcoming apparition was expected to give very favourable observing conditions in August–September 1989. But as of June, the comet had not been recovered.

We analysed the magnitude observations from 1919 (Fig. 2). Noting that in spite of naked-eye visibility there was no report of a conspicuous dust tail, we found it reasonable to trust the H_2O production rates derived using equation (4) and to use the resulting value of E . Later observations of the comet indicate that it is very dust-poor¹⁰. Unfortunately, E proved very difficult to measure—as seen in Fig. 2, post-perihelion observations are missing altogether and there is just a hint that maximum outgassing occurred several weeks before perihelion. Although the lack of post-perihelion data is partly the result of rapidly deteriorating observing geometry, we judged it likely that the brightness did fall off. Thus we arrived at an estimate of -0.26 ± 0.1 for E , and using a preliminary version of Fig. 1 this led us to an estimate of ΔP between -30 and -15 days. This being remarkably large, in absolute value, the failure to recover the comet using gravitational ephemerides seemed less surprising.

Just before our prediction¹¹ was published Helin¹² found the comet on a Schmidt plate taken at Mt Palomar on 4 July 1989. The position implied a correction of -15.6 days to the time of perihelion passage, roughly as we predicted. The comet was of magnitude 15 on recovery, indicating that it might have been found earlier if our analysis had been available. Our results may be useful in reducing the ephemeris uncertainties of other comets, particularly 'Halley-type' comets with $P \geq 20$ yr. In retrospect, the agreement between our prediction and the actual ΔP was somewhat fortuitous. The regression line in Fig. 1 actually implies ΔP in the range -6 to -15 days. The preliminary 1989

magnitude estimates indicate a perihelion asymmetry smaller than the one we derived for 1919, but this is still uncertain and might simply be an indication that the light curve has changed from 1919 to 1989. □

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Interstellar graphite in meteorites

Sachiko Amari*, Edward Anders*†, Alois Virag‡§
& Ernst Zinner‡

* Enrico Fermi Institute and Department of Chemistry,
University of Chicago, Chicago, Illinois 60637-1433, USA

† Physikalisches Institut der Universität, CH-3012 Bern, Switzerland

‡ McDonnell Center for the Space Sciences and Physics Department,
Washington University, St Louis, Missouri 63130-4899, USA

IN a continuation of our search for interstellar 'needles' in the meteoritic 'haystack'^{1,2}, we have now identified graphite grains, 1–4 µm in diameter, in the Murchison C2 chondrite. The interstellar origin of these grains is demonstrated by their ¹²C/¹³C ratio, which ranges from 0.09 to 16 times the Solar System value, and by the presence of the noble-gas component 'Ne-E(L)', nearly monoisotopic ²²Ne from the decay of ²²Na (with a half-life of 2.6 yr). The grains apparently formed in the outflows of novae and red giants, and demonstrate that graphite can form as a circumstellar condensate. Curiously, interstellar graphite is much rarer than interstellar microdiamonds (<2 p.p.m. compared to 400 p.p.m.) or even SiC (6–9 p.p.m.), although diamond is thermodynamically unstable relative to graphite. The main reason may be preferential destruction. Graphite is the third type of circumstellar grain that has become available for laboratory study.

The marker that guided us in the separations was Ne-E(L), which is strongly enriched in ²²Ne and is released on heating or combustion around 600 °C. Previous work^{3–5} has established that Ne-E(L) is located in a carbonaceous carrier (≡Cα) of density 2.0–2.5 g cm⁻³, grain size 1–10 µm, bulk δ¹³C = +340‰ (δ¹³C = [(¹³C/¹²C)_{sample}/(¹³C/¹²C)_{standard}] – 1) and estimated abundance ~5 p.p.m. In terms of the haystack analogy, however, the needle could no longer be recovered by burning the haystack with strong oxidants such as HClO₄, as this needle is not very resistant to oxidants.

The problem was to remove the abundant (~3 × 10⁴ p.p.m.) meteoritic kerogen by relatively gentle methods so as not to destroy Cα. After dissolving the silicates using HCl–HF, we partially oxidized the carbonaceous residue with Cr₂O₇²⁻ in 1M H₂SO₄, removed colloidal material, and then tried to enrich Cα by cautious oxidation with NaOCl and alkaline H₂O₂, followed by three successive density separations. Ne-E(L) appeared mainly in the fraction LFC (1.75–2.2 g cm⁻³; 1.0 p.p.m.), from which we isolated a coarse fraction LFC1 (nominally >1 µm, ~0.3 p.p.m.).

LFC mainly contains aggregates of ~0.1-µm spherules (Fig. 1a) and lesser amounts of dense particles. The aggregates broke up during ultrasonication and thus were largely removed by the size separation that produced LFC1. Planimetric analysis showed that this sample contains only ~10 wt% aggregates and ~90% dense particles of various morphologies (Fig. 1b), including 6% spheres (Fig. 1d), and even euhedral graphite crystals, with characteristic hexagonal outlines (Fig. 1c). The scanning electron microscopy/energy dispersive X-ray (SEM–EDX) spectrum of the latter grain shows that it is pure carbon.

Ion-probe analysis shows that the aggregates are a homogeneous population with normal carbon and slightly heavy nitrogen (Fig. 2). All are relatively rich in nitrogen (1–2%). In contrast, the dense grains (as well as some grains of undetermined morphology) generally have highly anomalous ¹²C/¹³C, from 0.09 to 16 times the Solar System value (Fig. 2). The highest ¹²C/¹³C ratio (1,440) far exceeds the highest ratio measured in meteorites (159; ref. 2) or carbon stars (97; ref. 6) but can, in principle, be produced in carbon stars of low metallicity⁷. Regrettably, nitrogen could not be reliably measured in the dense grains as it is much lower (<1/30) than in the aggregates, so that the analysis is usually dominated by nearby aggregates. One dense grain gives a distinctly heavy ¹⁴N/¹⁵N ratio of 85, whereas several others fall slightly above the mean for the clusters (Fig. 2), indicating either that their nitrogen is isotopically light or that adsorbed atmospheric nitrogen dominates.

Most of the dense grains are obviously exotic, as indicated by their large isotope anomalies. The origin of the aggregates is more ambiguous, because their only significant anomaly—a 30% enrichment in ¹⁵N—could have been produced by chemical processes in the Solar System. Regardless, it is now clear that the bulk carbon isotope value for Cα (¹²C/¹³C = 66) obtained by stepped combustion on a less enriched separate⁵ is simply the average of various exotic and normal components.

Structurally, the two classes also differ. In three of the dense grains examined by micro-Raman spectroscopy, lines characteristic of crystalline graphite but with varying degrees of disorder were observed, whereas no graphite lines but substantial fluorescence were observed in an aggregate, indicating an organic nature (B. Wopenka, personal communication). Further evidence for an organic nature is the high abundance of oxygen in the SEM–EDX spectrum, greatly exceeding that of the dense grains.

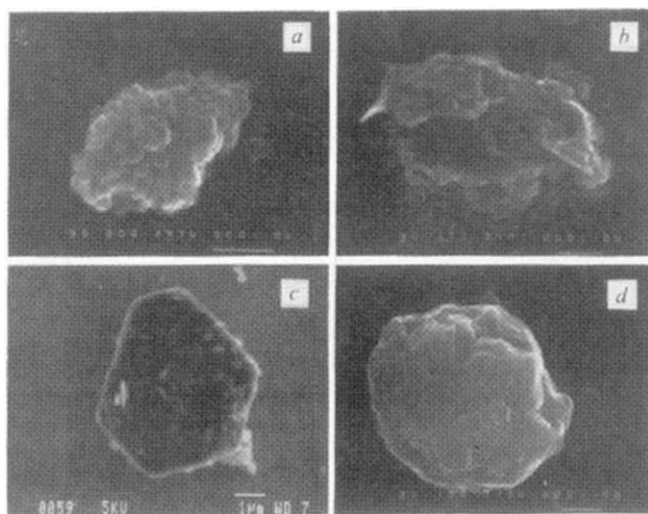


FIG. 1 SEM micrographs of carbon grains with different morphologies: a, Spherulitic aggregate; b, irregular platy grain with adhering spherules; c, hexagonal platy grain with small adhering spherules; d, compact sphere. Scale bar = 1 µm.

§ Present address: Institut für analytische Chemie, Technische Universität Wien, A-1060 Wien, Austria.

On mass-spectrometric analysis by stepped heating, both LFC and LFC1 turn out to be highly enriched in Ne-E(L), with a peak release at 700–800 °C and $^{22}\text{Ne}_E$ contents of 4,240 and $14,000 \times 10^{-8} \text{ cm}^3 \text{ g}^{-1}$. The latter value represents a 5×10^5 -fold enrichment over the bulk meteorite, and exceeds the highest previous values^{3,8} by a factor of 55–93. If Ne-E(L) is located only in some subclass of the dense grains, then its concentration in these grains must be higher still.

To resolve the isotope composition of the neon, we plotted the data on a three-isotope plot with a common denominator, using a logarithmic scale to expand the region near the origin (Fig. 3). The points lie along lines of slope unity from 'normal' neon towards the origin, suggesting that they are mixtures of normal Ne and pure ^{22}Ne , that is, Ne-E(L). From the argon and xenon data it seems that about 10% of the normal neon is Ne-A1,2 from microdiamonds ($\sim 0.5\%$ of the sample) and the rest is some typical planetary component of $^{20}\text{Ne}/^{36}\text{Ar} \approx 0.4$, with an isotopic composition presumably close to that of Ne-A in Fig. 3.

Sample LFC1 contains 3.3 times the concentration of Ne-E(L) of its parent LFC, whereas its siblings LFC2–LFC4 (the fractions $< 1 \mu\text{m}$, of densities $1.35\text{--}1.55 \text{ g cm}^{-3}$) contain only 0.01–0.02 times that amount. This suggests that the aggregates are not the carrier of Ne-E, as they are depleted in LFC1. Apparently, some subclass of the dense grains is the carrier. Moreover, the mass distribution of the dense grains must drop steeply below $1 \mu\text{m}$, as $< 1\%$ of the Ne-E is in the fractions $< 1 \mu\text{m}$.

At least one other type of interstellar grain seems to be hidden in the sample, judging from the presence of Kr-S and Xe-S, two components attributed to the astrophysical s-process (neutron capture on a slow timescale). Their only known carrier¹ is SiC, and their release temperature from LFC1 (1,150–1,450 °C) indeed resembles that from SiC, and is markedly higher than that of Ne-E(L) (Fig. 3). But their concentrations in LFC1 are 4.1 times and 0.25 times those of a typical SiC sample (35 and $76 \times 10^{-10} \text{ cm}^3 \text{ g}^{-1}$), although the Si/C ratios measured with the ion probe are only ~ 0.01 for the aggregates and < 0.001 for the dense grains. Evidently Kr-S and Xe-S in LFC1 are located in a minor refractory carrier of very high noble-gas content,

either SiC or some other phase, such as a highly retentive kind of graphite. One interesting possibility is that SiC grains served as condensation nuclei for amorphous carbon or graphite⁹. It seems that the search for the interstellar carriers of exotic noble gases continues to follow the previous pattern, in which the ultimate carrier apparently recedes into infinity as in the search for the innermost of a set of nested matryoshka dolls.

An intriguing hint of some unusual charged-particle irradiation is the presence of excess ^{21}Ne in LFC1. Depending on the assumed normal endmember, the sample contains $(0.67\text{--}1.16) \times 10^{-8} \text{ cm}^3 \text{ g}^{-1} \text{ }^{21}\text{Ne}$, compared to $0.25 \times 10^{-8} \text{ cm}^3 \text{ g}^{-1}$ in the bulk meteorite. The ^{21}Ne enrichment is largest in the 700 °C and 800 °C fractions, which contain most of the Ne-E (as shown by the deviation of these points from the mixing line in Fig. 3), suggesting that the excess ^{21}Ne is also located in carbon. But carbon is too light a target element to yield ^{21}Ne on proton bombardment, and as recoil from adjacent phases could at most have contributed $0.25 \times 10^{-8} \text{ cm}^3 \text{ g}^{-1} \text{ }^{21}\text{Ne}$, the excess probably represents recoils implanted during an earlier irradiation of dispersed dust and gas. This process is inefficient, because the ^{21}Ne recoiled out of silicate grains would be distributed over about 150 times the mass of gas and carbon dust (for material of solar composition). Even so, at typical cosmic-ray fluxes, the observed amount of ^{21}Ne could be implanted in less than the putative 500-Myr lifetime of refractory dust grains.

A principal conclusion from this work is that some graphite does form in stellar outflows¹⁰, despite the numerous difficulties that have been pointed out^{11,12}. An interesting clue is the one-time presence of ^{22}Na (the parent of ^{22}Ne). If it was trapped by condensation, then Na^+ may have helped nucleate graphite, despite arguments to the contrary^{10,13}. The $^{22}\text{Na}/\text{C}$ ratio in LFC1 is 7.5×10^{-8} , corresponding to $\sim 4 \times 10^4$ ^{22}Na atoms per $1\text{-}\mu\text{m}$ grain, and thus more than sufficient for nucleation. But perhaps the ^{22}Na was trapped by ion implantation only after the grains

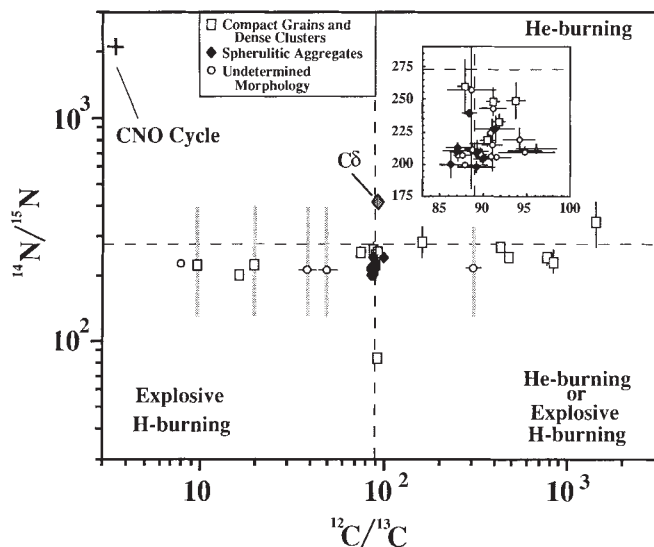


FIG. 2 Isotope ratios of carbon from Murchison LFC deviate from Solar System ratios (broken lines) according to morphology. Dense grains have highly variable carbon (factor of 180) but somewhat indeterminate nitrogen owing to interference from nearby nitrogen-rich aggregates (in five cases, designated by heavy error bars, nitrogen values could not be determined at all). The dominant nucleosynthetic processes are indicated for each quadrant, as is the composition of interstellar microdiamonds ('Cδ').

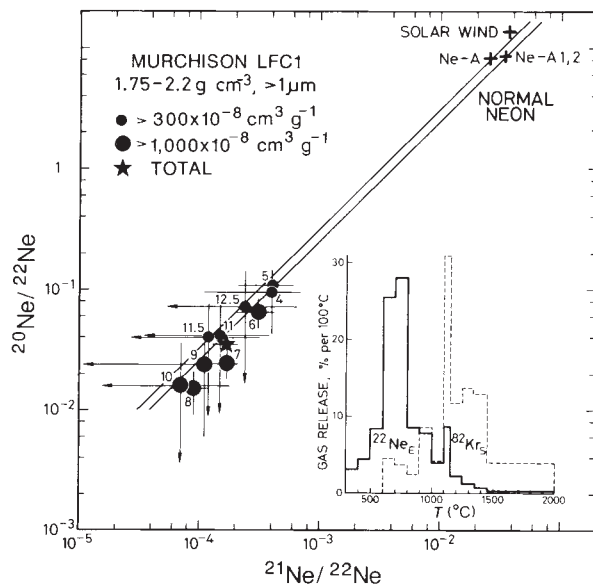


FIG. 3 Neon fractions extracted at progressively higher temperatures (numbers beside points indicate temperature in 10^2 °C) lie along a mixing line between normal Ne and pure ^{22}Ne (off-scale at lower left), suggesting that the neon is essentially a binary mixture of these components. Two interesting minor noble-gas components are also present. The 700 °C and 800 °C points stray from the mixing line, implying the presence of excess ^{21}Ne —presumably recoils trapped during a pre-solar cosmic-ray irradiation. Large amounts of s-process krypton and xenon are also present. As their release pattern peaks at higher temperatures than that of Ne-E (inset), they seem to be located in a refractory minor carrier of very high gas content.

had formed, in which case other ionized elements and nuclides should also be enriched. This possibility should be checked, but it is clear from the present data that ^{20}Ne and ^{21}Ne are not so enriched (Fig. 3).

Interstellar graphite turns out to be the rarest by far of the three types of circumstellar carbon isolated from meteorites to date. The abundance of dense graphite grains in the Murchison meteorite is ≤ 2 p.p.m., as estimated on the basis of LFC1 and the total Ne-E(L) content. This is strikingly less than the abundance of two other interstellar components, diamond (~ 400 p.p.m.) or SiC (~ 6 p.p.m.), and is only $< 1 \times 10^{-4}$ of the total carbon in the meteorite. Qualitatively, these amounts correlate with the chemical and mechanical resistance of these three carbon species. Amorphous carbon seems similarly rare; none of it showed up in our separations although the partly organic, presumably more reactive, aggregates survived. The upper limit on amorphous carbon is ≤ 10 p.p.m. if it is less reactive than the most resistant organic carbon in the meteorite, and perhaps ≤ 100 p.p.m. if it is more reactive.

The reason for the low abundance of interstellar graphite and amorphous carbon may be some combination of underproduction in stars¹² and selective destruction in interstellar space or the early Solar System. Such selective destruction has been invoked^{2,14} even for SiC, to explain its short cosmic-ray exposure age and low abundance in meteorites². Another possibility is preferential formation of diamond owing to kinetic¹⁵ or thermodynamic¹⁶⁻¹⁸ factors. Although the thermodynamic stabilization of microdiamonds seems plausible on the basis of smaller surface energy^{16,17} or formation energy of vacancies¹⁸, it has not yet been confirmed experimentally. Also, such stabilization is hard to reconcile with the consistent formation of soot rather than microdiamonds in terrestrial fires.¹⁹

Because of the complexity of our sample we can make only a few general comments about its stellar origins. The short, 2.6-yr half-life of ^{22}Na , the parent of Ne-E, requires rapid formation under explosive conditions, such as those in a nova²⁰, and rapid trapping in grains. Indeed, novae are believed to produce graphite grains of only slightly smaller size^{21,22}. Only one of the grains in Fig. 2, however, shows the ^{15}N enrichment expected for a nova. Perhaps only few of even the dense grains contain Ne-E(L), or they condensed under conditions unfavourable for trapping of nitrogen. Asymptotic giant branch stars are a potentially much larger source, but it is not certain¹⁰ that they can condense large graphite grains in their outflows. A clearer picture may emerge when other elements are measured in the dense grains.

Note added in proof: Subsequent work²³ has shown that only the round grains (Fig. 1d) have isotopically anomalous carbon. Thus, soot-like spherules²⁴ are a significant component of interstellar graphite. □

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Oxyfunctionalization of alkanes with hydrogen peroxide on titanium silicalite

D. R. C. Huybrechts, L. De Bruycker & P. A. Jacobs

Laboratorium voor Oppervlaktechemie, Katholieke Universiteit Leuven, Kardinaal Mercierlaan 92, B-3030 Heverlee, Belgium

BECAUSE of the chemical inertness of alkanes, the introduction of functional groups into them by oxidation has tended to require severe and thus unselective conditions. The homogeneous and heterogeneous catalysts used for alkene oxidation are ineffective for alkanes^{1,2}. Cleavage of carbon-carbon bonds in the presence of transition metal complexes effects mainly complete oxidation to acids², although some selectivity is occasionally possible³. Oxyfunctionalization without C-C bond cleavage has also been achieved^{2,4,5}, and high selectivity may be obtained from natural or synthetic metalloporphyrin systems^{6,7}. Here we show that titanium silicalite, a microporous crystalline Ti-silica⁸, is able to activate secondary and tertiary carbon atoms in alkanes, so that they may be oxidized to alcohols and subsequently to ketones using aqueous H_2O_2 . The catalyst has low regioselectivity for alcohol formation but high reactant selectivity in the formation of ketones.

Several rather specific processes are available for alkane oxyfunctionalization. Vanadylphosphates are selective catalysts for the conversion of butane with molecular oxygen into maleic anhydride³. Catalytic conversion of cyclohexane into cyclohexanol and cyclohexanone using a cobalt catalyst is an established industrial process², and hydrocarbon oxidation catalysed by a heteropolyanion was reported recently⁴. Oxidation of alkanes to alcohols and ketones has been reported⁵ using a Pd-Fe zeolite in an O_2/H_2 atmosphere. The method that we report here confirms the potential of zeolite catalysts in processes of this sort.

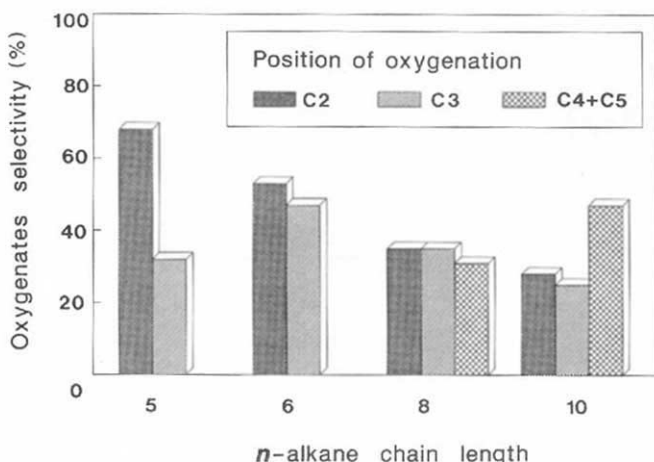


FIG. 1. Oxyfunctionalization of *n*-alkanes at different positions in the alkane chain by H_2O_2 (aq) over titanium silicalite.

TABLE 1 H₂O₂ yields

Substrate	H ₂ O ₂ yield (%)
<i>n</i> -pentane	68
<i>n</i> -hexane	70
<i>n</i> -octane	65
<i>n</i> -decane	56
2-methylpentane	59
3-methylpentane	58
2,2-dimethylpentane	50

H₂O₂ yield is H₂O₂ used for alkane oxidation divided by total amount of H₂O₂ used.

Taramasso *et al.*⁸ reported the synthesis of titanium silicalite (TS-1), a titanium-containing silicalite-1 zeolite. TS-1 is known to catalyse oxidations of unsaturated hydrocarbons with aqueous H₂O₂, such as the epoxidation of alkenes, the oxidation of alcohols to ketones, and the hydroxylation of aromatics⁹⁻¹¹.

Titanium silicalite was prepared hydrothermally according to a published procedure⁸. Its X-ray diffraction, infrared and electron microscopy characteristics are in agreement with those reported⁸ for TS-1. Typical oxidation experiments were conducted at 373 K for 3 h, with 400 mg of TS-1, 310 mmol of alkane,

210 mmol of a 35% aqueous solution of H₂O₂ and 0.81 mol of acetone. The conversion of H₂O₂, as determined by cerimetric titration, is >90% in all cases. The oxidation products are mostly alcohols and ketones. The yields, based on carbon, add up to 100% within the accuracy of the gas chromatographic analysis.

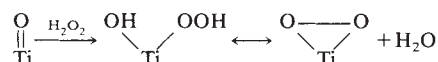
Table 1 gives the efficiency of H₂O₂ use in alkane oxidations over TS-1. For *n*-alkanes as well as for hexane isomers, the H₂O₂ efficiency is high but decreases with increasing dimensions of the organic substrate. Because of the pore geometry of TS-1, bulkier substrates have lower oxidation rates and the main side reaction—decomposition of H₂O₂ to O₂—is therefore more competitive.

Figure 1 shows the selectivity for the different carbon positions along the *n*-alkane chains. The total amount of oxygenated products is distributed in an almost statistical way among the different secondary carbon atoms. Even at low conversions, the alcohol selectivity is never 100%, so the slowest step in the reaction, the formation of alcohols from alkanes, is not very regioselective.

Figure 2 shows that in the ketone fraction the selectivity for 2-alkanones increases with the chain length of the substrate. This selectivity shows that the oxidation of 2-alkanols to 2-alkanones is favoured, which can be explained by the geometric constraints imposed on the molecules by the intracrystalline volume of the molecular-sieve structure. Similar distributions are observed when *n*-alkanes are isomerized to their methyl isomers over the acid alumino-silicate homologue of TS-1 (ref. 12).

In the oxyfunctionalization of hexane, and 2- and 3-methylpentane, oxidation occurs selectively at the tertiary C—H (Fig. 3). Oxidation of primary C—H bonds was below detection. Alkane oxyfunctionalization therefore follows the usual reactivity order: tertiary C—H > secondary C—H ≫ primary C—H. For 3-methylpentane the reactant selectivity effect, which requires preferential formation of the C₂ ketone, is overcompensated by the high reactivity at C₃ for alcohol formation.

No extensive mechanistic studies of the alkane oxidation catalysed by titanium silicalite have so far been made, but the surface titanyl (Ti=O) group is believed to be the basic unit of the active site. Hydroxyhydroperoxo or peroxo complexes formed on the titanyl group by reaction with hydrogen peroxide are believed to be the oxygen donors. The formation of such complexes is represented in scheme 1:



Evidence for the formation of peroxo complexes is found by absorption of H₂O₂ on TS-1 followed by vacuum drying. This treatment causes the disappearance of the 960-cm⁻¹ infrared band, ascribed to the surface titanyl group⁸, and the appearance of a 425-nm absorption band in the visible part of the spectrum. This absorption is characteristic for titanium complexes with peroxo ligands¹³. Heating of the H₂O₂-treated titanium silicalite leads to the decomposition of the Ti peroxo complexes with reformation of the titanyl group (the 425-nm band disappears and the 960-cm⁻¹ absorption band reappears).

Considering the inertness of saturated hydrocarbons, their oxidation probably requires a homolytic mechanism, giving rise to radical intermediates. Such a mechanism is tentatively represented in scheme 2, by analogy with the mechanism of the stoichiometric alkane oxidations by vanadium peroxo complexes¹⁴:

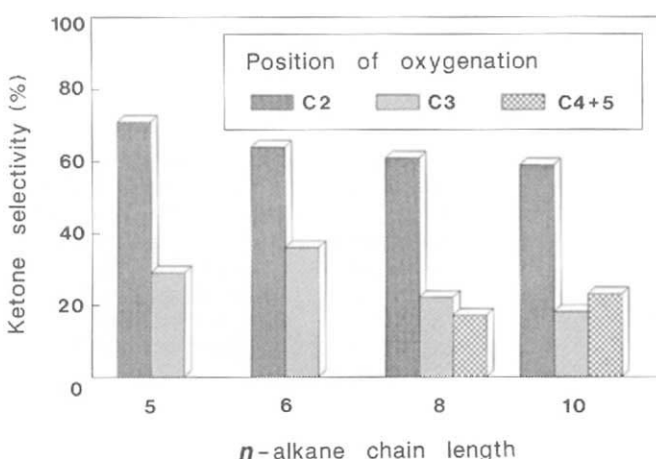
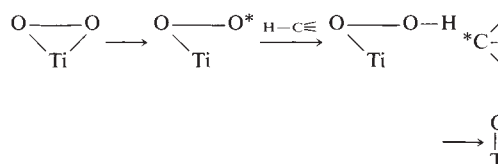


FIG. 2 Selectivity in the alkanone fraction of the oxygenates obtained by oxyfunctionalization of *n*-alkanes with H₂O₂ (aq).

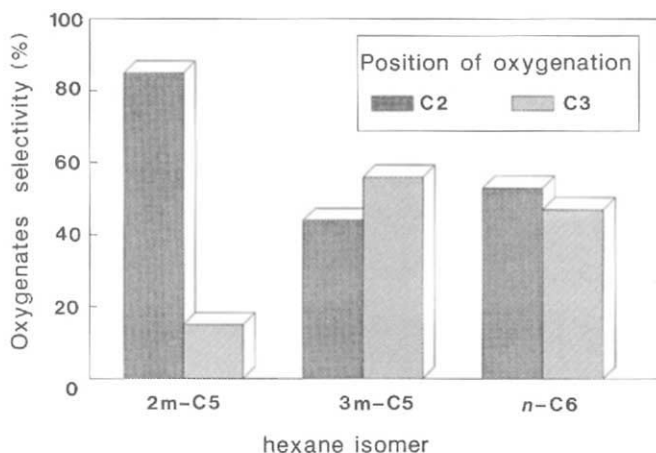


FIG. 3 Oxyfunctionalization of hexane isomers at different positions in the alkane chain by H₂O₂ (aq) over titanium silicalite. (2m-C5, 2-methylpentane; 3m-C5, 3-methylpentane).

In this scheme, the reactivity of the titanium peroxo complex is attributed to the generation of an open diradical form, followed by hydrogen abstraction from the alkane to give a carbon radical. Recombination with the hydroxyl radical from $\text{Ti}-\text{O}-\text{OH}$ yields the alcohol and the titanyl group.

After the completion of this work Tatsumi *et al.*¹⁵ reported on this topic. The best turnovers mentioned by these authors for *n*-hexane are 35 mol per mol Ti. Here values of >1,000 are obtained for the same substrate and over the same period of time. □

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Role of sub-micrometre particles in the ocean

Koike Isao*, Shigemitsu Hara†‡, Kazuki Terauchi* & Kazuhiro Kogure*

* Ocean Research Institute, University of Tokyo, Nakano, Tokyo, 164, Japan

† Graduate School of Science and Technology, Kobe University, Kobe, Japan

PARTICULATE matter plays an important part in biogeochemical cycles in the ocean; as particles settle out of the water column, they carry with them carbon and other adsorbed chemicals. Because particles of sub-micrometre size (at the boundary between 'particulate' and 'dissolved' materials) do not have appreciable settling rates in natural waters, they have generally been considered unimportant in the downward flux of particles and the nature and origin of the particles are largely unknown^{1–5}. Here we present results from epifluorescence microscopy and from particle counting which have allowed us to determine the vertical distribution of sub-micrometre particles (size range 0.38–1 μm). We find that >95% of these particles are non-living and occur in the upper layers of the ocean (50 m) in concentrations of the order of 10 million per millilitre. Many of the non-living particles seem to be fragile and flexible, and seem to have a high water content and to be composed largely of organic material. The size distribution of these sub-micrometre particles leads us to conclude that a significant portion (at least 10%) of 'dissolved' organic material may in fact be in the form of these small particles, as suggested by Sharp⁶.

Water samples were collected using a rosette multi-sampler during the KH-86-3 cruise⁷ of the RV *Hakuho Maru* to the northern North Pacific Ocean. Surface-water samples used for experimental procedures were obtained from coastal areas in Japan with an acid-washed bucket after several rinses. Samples,

TABLE 1 Size fractionation of sub-micrometre particles (0.38–1.0 μm) and bacteria in the coastal surface waters using two types of filters

Size fraction (μm)	Bacterial number (ml^{-1})	Particle number (0.38–1.0 μm) (ml^{-1})	Particle volume (0.38–1.0 μm) ($\mu\text{m}^3 \text{ml}^{-1}$)
Off Tokyo Bay*			
Net			
<5	7.0×10^5 (100%)†	1.2×10^7 (100%)	6.2×10^5 (100%)
Nuclepore filter			
<1	5.1×10^5 (73%)	1.0×10^7 (84%)	4.8×10^5 (77%)
<0.4	2.4×10^5 (34%)	7.6×10^6 (62%)	3.3×10^5 (53%)
<0.1	4.0×10^3 (0.6%)	5.3×10^6 (43%)	2.2×10^5 (35%)
<0.05	3.8×10^3 (0.5%)	3.3×10^5 (2.8%)	1.5×10^4 (2.4%)
Millipore filter			
<0.45	8.9×10^4 (13%)	3.3×10^6 (27%)	1.4×10^5 (23%)
<0.1	1.3×10^4 (1.9%)	2.3×10^6 (19%)	9.5×10^4 (15%)
<0.05	4.8×10^3 (0.75%)	1.7×10^6 (15%)	6.9×10^4 (11%)
Otsuchi Bay‡			
Net			
<10	1.3×10^6 (100%)	3.3×10^7 (100%)	1.6×10^6 (100%)
Nuclepore filter			
<1	5.8×10^5 (45%)	3.5×10^7 (105%)	1.6×10^6 (100%)
<0.4	2.3×10^5 (18%)	2.5×10^7 (74%)	1.0×10^6 (64%)
<0.1	1.5×10^4 (1.2%)	1.7×10^7 (53%)	7.1×10^5 (45%)
<0.05	2.0×10^4 (1.5%)	7.2×10^4 (0.22%)	7.2×10^4 (0.46%)
Millipore filter			
<0.45	—	1.5×10^7 (44%)	—
<0.10	—	7.0×10^6 (8.2%)	—

* Off Tokyo Bay: location (35°08'N, 139°44'E).

† Numbers in parentheses represent the percentage fraction after the sequential filtration of net treated seawater samples (100%).

‡ Otsuchi Bay: location (39°20'N, 141°56'E).

which were kept at 4 °C, were handled so as to minimize any physical agitation and measurements were made within 12 hr of collection. No significant change in the number of bacteria or sub-micrometre particles was observed during this period. For counting the sub-micrometre-sized particles, an Elzone Monitor Particle Counter 80 XY (Particle Data Inc. USA), which has 128 channels of size distribution, was used with a 12- μm -orifice counting tube. Although the counting tube has the nominal capability of counting particles with a spherical diameter of 0.34–2.02 μm , the practical limit of the minimum measurable size was 0.36–0.38 μm , depending on the electronic noise. The calibration of the instrument and dilution of the samples has been described elsewhere⁸.

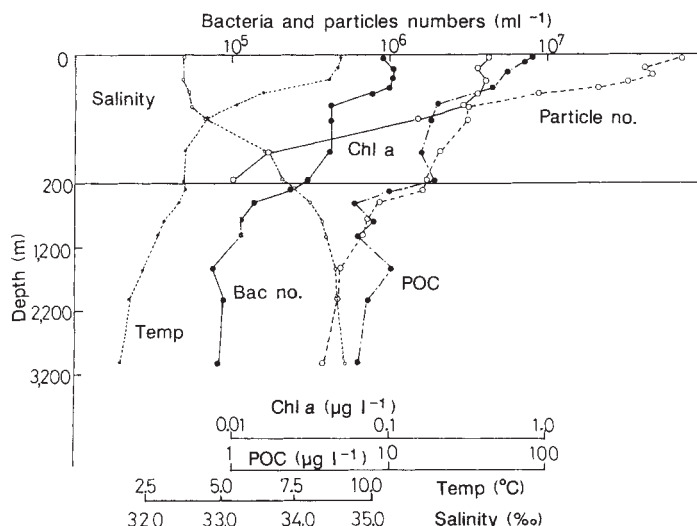


FIG. 1 Vertical profiles of number of sub-micrometre particles, bacterial number, chlorophyll *a* (chl *a*), particulate organic carbon (POC), temperature and salinity at Station F' (52°55.9'N, 144°55.9'W).

† Present address: 'Sogo-Kagaku' Environmental Assessment Consultation Company, Nojinbashi, 1-1-7, Chuo-ku Osaka, 540, Japan.

TABLE 2 Number of sub-micrometre particles (0.36–1.0 μm) and microorganisms during seawater culture

(Day)	Treatment 1*		Treatment 2†		Treatment 3‡	
	Bacterial number	Particle number (ml^{-1})	Bacterial number	Particle number (ml^{-1})	Bacterial number	Particle number (ml^{-1})
Off Tokyo Bay surface water						
T=0	6×10^3	2.6×10^5	1.5×10^4	3.8×10^5	ND§	ND
T=29	7.5×10^6	5.9×10^6	2.0×10^5	4.6×10^6	ND	ND
T=55	7.8×10^6	1.0×10^7	6.6×10^5	1.4×10^7	ND	ND
Sagami Bay surface water						
T=0	3.9×10^4	2.6×10^5	1.2×10^4	2.6×10^5	2×10^4	1.8×10^5
T=32	4.0×10^6	3.7×10^6	4.0×10^5	5.4×10^6	2×10^4	2.4×10^5
T=68	3.6×10^6	4.5×10^6	1.8×10^5	2.4×10^6	2×10^4	3.4×10^5

* Anopore (0.02 μm) filtered sea water was enriched with 1% (v/v) of GF/F (the cut-off: 0.6 μm) filtered sea water. Incubation was in the dark at room temperature.

† Anopore (0.02 μm) filtered sea water was enriched with 1% (v/v) of 5 μm net filtered sea water. Incubation was in the dark at room temperature.

‡ Anopore (0.02 μm) filtered sea water was enriched with 1% (v/v) of 5 μm net filtered sea water plus 2% (final concentration) of formalin. Incubation condition was in the dark at room temperature.

§ Not determined.

Vertical profiles of sub-micrometre particles (total number in the range 0.38–1.0 μm diameter) were compared with physical and other biological parameters at the station (52°59.9' N, 144°55.9' W) in the northern North Pacific Ocean (Fig. 1). The number of sub-micrometre particles was 5×10^7 – $8 \times 10^7 \text{ ml}^{-1}$ in the top 40 m and decreased to 2×10^6 at a depth of 200 m. In the upper 200 m, >95% of the measured particles were <0.6 μm in size. Bacterial populations are the major portion of living particles in this size range^{1,9,10}. As the Elzone Counter counts all the particles in the above-mentioned size range, the fraction of living particles in the total sub-micrometre particles can be estimated from simultaneous bacterial counts obtained by epifluorescence microscopy¹⁰. The bacterial number was $1 \times 10^6 \text{ ml}^{-1}$ in the surface layer and decreased to 4×10^5 at 200 m. Regression analysis using the data obtained from three pelagic stations (52°59.9' N, 144°55.9' W; 53°30.5' N, 177°31.7' E; 51°03.6' N, 170°58.1' E) in the summer of the northern North Pacific (upper 200 m) indicated a significant linear correlation between the number of sub-micrometre particles and bacterial number ($r = 0.769$; $n = 24$). The total number of sub-micrometre particles, however, was on average 30 times higher than that of bacterial counts of similar size, indicating that >95% of sub-micrometre particles of this size is non-living. This estimate is rather conservative, as some bacteria are <0.38 μm in size. The difference between bacterial and total sub-micrometre particle number decreased with depth, although a 4–6 times difference was still observed at the deep layer (2,000–3,000 m). Vertical profiles of sub-micrometre particles also correlated to some extent with other measured parameters, such as chlorophyll *a* ($r = 0.641$; $n = 24$) and particulate organic carbon collected by GF/F filters (nominal cut-off: 0.6 μm ; $r = 0.691$; $n = 11$), suggesting the biological origin of these particles.

Size fractionation of bacteria and total sub-micrometre particles of surface coastal waters using two types of filters showed a distinct difference in the efficiency of retention (Table 1). Both Nuclepore and Millipore filters of 0.1- μm pore size retained ~99% of the bacteria (gravity filtration with <100-mm water height), which is consistent with previous reports^{9,10}. Almost half of the total sub-micrometre particles (0.38–1.0 μm) passed through a 0.1- μm Nuclepore filter, but most were collected with the 0.05- μm Nuclepore filter. Depending on both the material of the filters and their pore size, retention of the sub-micrometre particles was different; this is consistent with observations for other particles¹¹. The apparent contradiction, that is, retention of only half of the minimum 0.38- μm sized particles by the 0.1- μm Nuclepore filter, may be explained if we assume the flexible character of the non-living sub-micrometre particles as discussed below. Short-time sonication (30 s) of the samples,

which had no significant effect on bacterial counts, caused a large decrease in the number of sub-micrometre particles compared with non-sonicated samples, although reduction to 2% for samples prefiltered with a 0.4- μm Nuclepore filter was much larger than that for non-filtered samples (20%). Larger-sized particles might be a possible source of sub-micrometre particles through disaggregation caused by sonication. Ultra-centrifugation (100,000g for 2 hr) of the seawater samples, on the other hand, had almost no effect on the number of particles <0.6 μm in diameter, indicating a very small density difference between the sub-micrometre particles and the surrounding water. From the results of physical treatments of the sea water, some characteristics of marine sub-micrometre particles can be inferred. Strong agitation, such as sonication, results in a reduction in number instead of an increase, thus reducing the likelihood of artificial fragmentation of marine-snow-type detritus being the major source of the observed sub-micrometre particles^{12,13}. Most of these sub-micrometre particles, therefore, are actually present as non-living particles in the ocean with a fragile and flexible nature and possibly with a very high water content. With transmission electron microscopy, we found many amorphous aggregates (~6–8 times the number of bacteria) together with rigid shaped particles, such as bacteria and solid fragments of planktonic organisms. We suggest that these amorphous aggregates originated from the coagulation of sub-micrometre particles described above.

We also found that Anopore filter (0.02 μm) removed most of the sub-micrometre particles from sea water ($\leq 0.5\%$ remained after filtration). The biological origin of the non-living sub-micrometre particles was indicated by incubation (1–2 months) of Anopore-filtered bacterial seawater culture enriched with <5- μm seawater fraction (Table 2). The presence of a large-size fraction (0.6–5.0 μm) seems to be essential for the production of non-living particles in this system, because the number of sub-micrometre particles and bacteria was roughly the same during incubation of the bacterial fraction, that is, most of the particles produced were bacteria (Treatment 1 in Table 2). A large increase in number of flagellates (2×10^4 – $3 \times 10^5 \text{ ml}^{-1}$; size: 1.5–2.0 μm) was found after 1 month of incubation in the sample from off Tokyo Bay, suggesting that grazing of bacteria by flagellates is responsible for the formation of these sub-micrometre particles. No increase in sub-micrometre particles or bacterial number was observed when the sample was treated with formalin (Treatment 3 in Table 2).

Most of the sub-micrometre particles <0.6 μm in size passed through a GF/F glass fibre filter, which is commonly used to separate total organic particles from 'dissolved' organics in the ocean. If we use the reported concentrations of dissolved organic carbon (1.0–3.0 mg C l^{-1}) in the upper open ocean, which include recent measurements using the high-temperature method^{14,15}, a significant portion ($\geq 10\%$) of 'dissolved' organic material may be in the form of sub-micrometre particles, as suggested previously⁶. Although the chemical composition and exact origins of these particles are not clear at the moment, their presence and one possible mechanisms of their formation are important in evaluating the nature of 'dissolved' materials in the ocean. The importance of these sub-micrometre particles should also be stressed when considering chemical and biological processes and optical aspects in the upper ocean, as they are numerous and provide a large solid/liquid interface (0.2–0.5 $\text{cm}^2 \text{ ml}^{-1}$). □

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Influence of hydroxyl-bearing minerals on the isotopic composition of ice from the basal zone of an ice sheet

R. Souchez*, M. Lemmens*, R. Lorrain*, J.-L. Tison*, J. Jouzel† & D. Sugden‡

* Faculté des Sciences, CP 160, Université Libre de Bruxelles, B-1050 Brussels, Belgium

† Laboratoire de Géochimie Isotopique, CEN, Saclay, F-91191 Gif-sur-Yvette, France, and DLPC, Laboratoire de Glaciologie et de Géophysique de l'Environnement, BP 96, 38402 Saint-Martin-d'Hères Cedex, France

‡ Department of Geography, University of Edinburgh, Edinburgh EH8 9XP, UK

ONE of the important questions in ice-core studies is the extent to which isotope measurements made on ice loaded with bedrock debris can be trusted when interpreting the climate record from ice-core data. Here we show that the isotopic characteristics of basal ice containing debris from beneath the Greenland ice sheet can be understood only if isotope exchange between hydroxyl-bearing minerals and water is taken into account. Sub-glacial abrasion of clays and micas increases the number of broken bonds at the border of the mineral structure, promotes cleavage and so increases the rate at which these minerals exchange oxygen and hydrogen isotopes with water. One implication of our results is to call into question the recent suggestion¹ that the basal ice of the Greenland ice sheet is 'superimposed ice', and that therefore extensive melting must have taken place during the last interglacial. The enrichment in ¹⁸O of the basal ice, thought to be acquired during the formation of the superimposed ice, may in fact be the result of isotope exchange with hydroxyl-bearing minerals.

Different ice facies having different isotopic characteristics may be recognized in basal ice, the main distinction being between an upper dispersed facies and a lower stratified facies^{2,3}.

The dispersed ice facies examined in this study has been recognized in different localities in West Greenland: along both lateral flanks of Russell Glacier, a small outlet glacier from the ice sheet ~25 km east from Søndre Strømfjord airport, and in its frontal zone; along an ice ramp representing a lobate margin at Qigssertaq (Fig. 1). This dispersed facies is probably equivalent to the 'black ice' found by Reeh and Thomsen⁴ northeast from Jakobshavn in the Pâkitsup area. The dispersed facies is composed of clear, bubble-poor ice with a crystal size ~1.5-3.5 cm mean diameter in thin section; it contains suspended lenticular nodules of debris often <1 cm in diameter but occasionally reaching 8 cm (clotted ice). The debris in the nodules is an aggregate of silt- and clay-sized particles. The mineral assemblage is consistent with an origin from gneissic bedrock with >40% comprising mica⁵. Grain-size distributions of the debris in the nodules show a mode in the fine-silt fraction due to abrasion⁶. By contrast, the debris in the lower stratified facies has two peaks in the gravel and coarse silt/fine sand fractions, respectively; these are typical of processes of sub-glacial crushing⁶. At Qigssertaq, where no folds are present, the

dispersed facies directly above the stratified facies is a few metres thick.

Clausen and Stauffer⁷ have analysed two ice cores drilled at the ice-sheet margin in West Greenland in the Camp 3 area northeast of Jakobshavn. The two drill sites were 300 m and 700 m from the marginal moraine respectively and reached the floor at depths of 46 m and 92 m, respectively. The ice stratigraphy was preserved at the two sites and ¹⁸O records reveal the characteristic shift for the Pleistocene-Holocene transition. Temperature profiles were measured in the bore holes. In both localities, beneath an upper layer of cold ice, the temperature reached the melting point at 30 m depth. Ice at the base of the ice sheet in this marginal area is thus temperate. It is believed that in a similar position and flow régime, further south, in the Søndre Strømfjord area, a similar temperature profile would be obtained. Therefore, basal ice appearing along the ice-sheet margin at Qigssertaq or at Russell Glacier is, or has most probably been, at the pressure melting point at depth.

The ice cores retrieved during the summer of 1987 were kept frozen between the sampling site and the cold room in Brussels. The ice samples were cut with a microtome and allowed to melt completely. The melt water was transferred into a 2-ml glass vial. Care was taken to minimize the possibility of exchange between melted ice and air and to prevent evaporation. Samples were analysed at the Centre d'Etudes Nucléaires de Saclay in France by twin mass spectrometers allowing co-isotopic measurements on a single liquid drop. The δD and $\delta^{18}O$ obtained are expressed in parts per mil (‰) relative to Vienna standard mean ocean water ($\delta D = [(^2H/^1H)_{sample}/(^2H/^1H)_{standard}] - 1$; $\delta^{18}O$ is defined similarly). Accuracy of the measurements is $\pm 0.5\%$ for δD and $\pm 0.1\%$ for $\delta^{18}O$.

Glacier ice samples are represented on Fig. 2. They are aligned on a regression line having the equation $\delta D = 8.22 \delta^{18}O + 13.01$, and a correlation coefficient of 0.996. This is very close to the meteoric-water line and can be considered to be the result of a precipitation effect. Three points on this line are grouped on its less negative side: one is local snow and the two others are thought to be superimposed ice. Excluding these three points the range in δ -values between the less negative and the most negative glacier ice sample is about 70‰ in δD and 9‰ in $\delta^{18}O$.

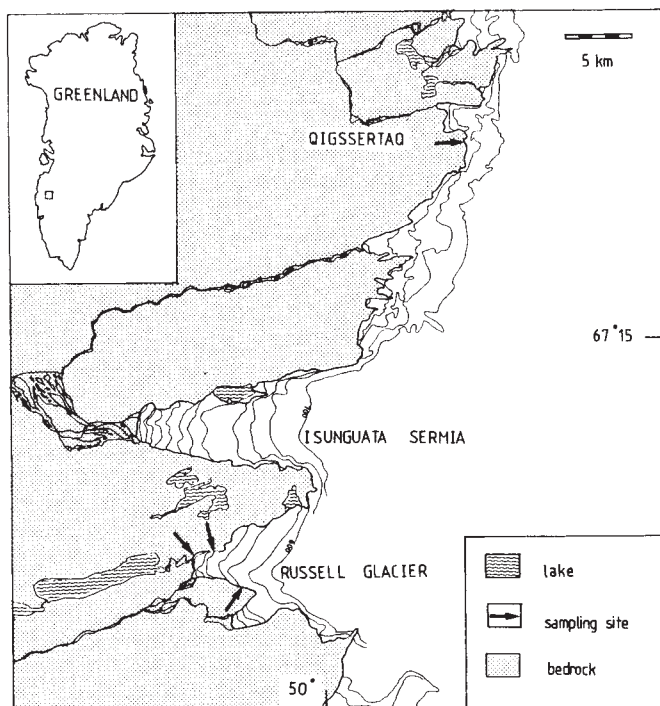


FIG. 1 The western margin of the Greenland ice sheet near Søndre Strømfjord.

This shift probably indicates a Pleistocene age for the most negative values.

Ice samples from the dispersed facies have two kinds of distributions (Fig. 2). First, some samples are grouped on or close to the precipitation slope. The others deviate significantly from it and are more-or-less aligned. Calculated regression lines are: (1) $\delta D = 4.83 \delta^{18}O - 94.71$ for Qigssertaq with a correlation coefficient of 0.990; and (2) $\delta D = 3.71 \delta^{18}O - 132.00$ for Russell Glacier with a correlation coefficient of 0.968. These regression lines cannot be considered as freezing slopes. Indeed, if in accordance with findings^{8,9} the δ -values at the intersections of the regression lines and the precipitation slope are the initial δ -values of the water undergoing freezing^{8,9}, then the calculated freezing slopes are much too high (>5.35). Therefore freezing cannot explain the special distributions displayed on a δD - $\delta^{18}O$ diagram by ice samples from the dispersed facies.

An alternative explanation involves the debris in the ice. Some ice cores from the dispersed facies show a zone of thin laminated debris layers interrupting the clotted-ice sequence, probably as a consequence of shear. Such a zone in a core from Qigssertaq about 2.0 cm thick was investigated in detail with a microtome in a cold room (ice layer A). Figure 3 shows results of the analyses. Considerable enrichment in heavy isotopes occurs when the debris/ice ratio is particularly high. This raises the question of the interaction between fine particles and the isotopic composition of the ice with which they are in contact.

The presence of both hydrogen and oxygen in the crystal structure of hydroxyl-bearing minerals makes them a potential source of stable isotopes for exchange reactions between minerals and water. In the West Greenland sites investigated,

biotite, amphibole and chlorite are the principal hydroxyl-bearing minerals, with mica being the most abundant in the fine fraction of the dirt included in the ice.

In the determination of the stable isotope composition of hydroxyl-bearing minerals, interlayer and adsorbed water is removed before analysis because this water achieves an almost complete isotope exchange with atmospheric water vapour in a few hours¹⁰. It is generally found that the $\delta^{18}O$ values of hydroxyl-bearing minerals inherited from parent rocks range between +7‰ and +27‰ with clays more positive than the micas. The hydrogen isotope data show a similar range of δ -values for micas and clays, the δD values lying between -40‰ and -100‰. Savin and Epstein¹⁰ have found that the isotopic compositions of clays of low-temperature origin are more-or-less aligned on a line parallel to the meteoric-water line. The clays are enriched in ^{18}O relative to the water but impoverished in deuterium.

In a study of oxygen isotope exchange between illite and water at 22 °C, James and Baker¹¹ indicate that exchange is controlled by the accessibility of water to the interlayer cation region. Illitic material, which shows expandable interstratification, may readily undergo significant post-formational isotope exchange with its aqueous environment. In the suspensions considered, a large volume of solution relative to the amount of clay was used so that the oxygen isotopic composition of the solution would remain essentially constant throughout the experiment while the $\delta^{18}O$ of the clay changed. O'Neil and Kharaka¹² have found that hydrogen isotope exchange occurs by a mechanism of proton exchange, independent of the slower process of ^{18}O exchange. For micas, the problem of accessibility to isotope exchange with water has also been recognized¹³, a breakdown of the structure of the mineral favouring exchange. Grim¹⁴ indicates that grinding of micas and clay minerals considerably increases their cation exchange capacity either by increasing the number of broken bonds at the border or edge of the mineral or by favouring cleavage. The cation exchange capacity of the hydroxyl-bearing minerals measured in the silt fraction of the dirt in ice layer A is 20 meq per 100 g, which is much higher than the 'normal' value of 3 meq per 100 g given for biotite less than 150 μm (ref. 14).

If the water volume relative to the amount of hydroxyl-bearing minerals is not large, the isotopic composition of the water is likely to be affected markedly by a process of isotope exchange. For example, if ground material comes into contact with melt water from Pleistocene ice, the water will become enriched in heavy isotopes. In the core containing ice layer A, minute amounts of water were present between ground particles and ice when the melting point was reached at depth and the cutting of the sample by the microtome has certainly resulted in further grinding. The original isotopic composition of the ice has therefore been modified. This is the reason for the correspondence between more positive δ -values in the melt water and a higher

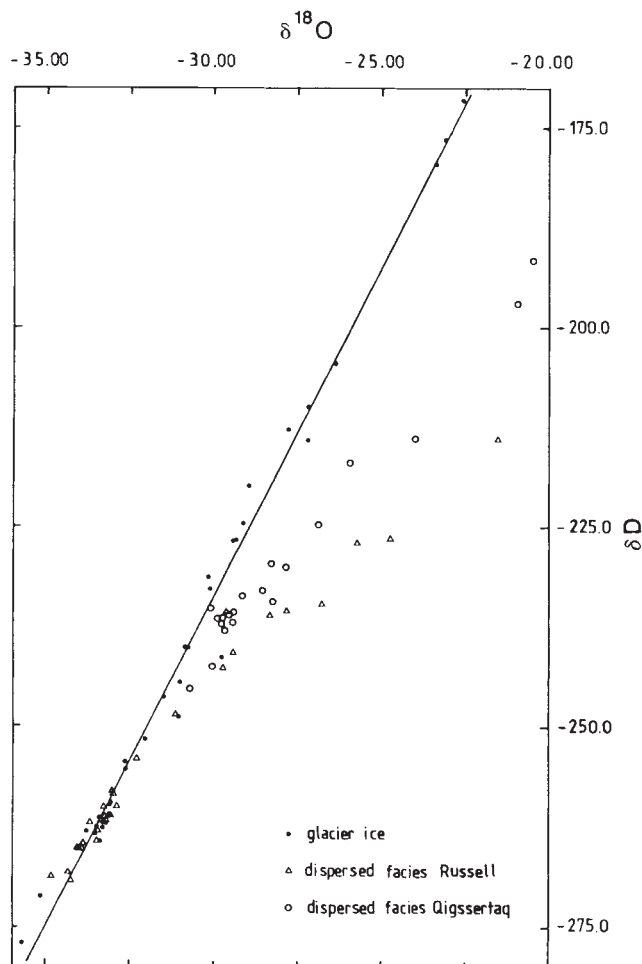


FIG. 2 δD - $\delta^{18}O$ diagram of samples from glacier ice and from the dispersed facies. The equation of the line is $\delta D = 8.22 \delta^{18}O + 13.01$.

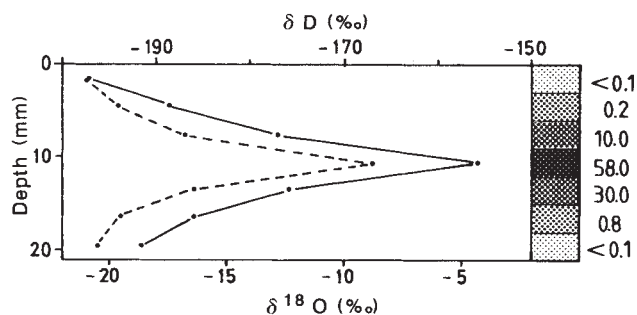


FIG. 3 Isotopic composition and debris/ice ratios in a thin zone of laminated debris layers at Qigssertaq (ice layer A). The solid line refers to the δD scale, the dashed line to the $\delta^{18}O$ scale. The measured debris/ice ratios of the samples are given (wt%) on the right. Shading is a visual representation of the debris concentration.

debris/ice content and for the large range of δ -values within the 2-cm-thick ice layer A zone. The difference between the $\delta^{18}\text{O}$ value of the ice with the highest debris concentration (-8.8%) and that of the bulk ice (-20.9%) can therefore be considered as being the result of isotope exchange with hydroxyl-bearing minerals. Calculated ^{18}O loss per g of mineral is of the same order of magnitude as ^{18}O increase per g of clay, which can be computed from James and Baker's experiments¹¹. This indicates the likelihood of the invoked process.

As indicated above, ice from the dispersed facies is likely to have been at the melting point at depth; water could thus have been present, accounting for the almost complete disappearance of gas bubbles. The characteristics of this ice reflect a situation associated with small-scale regelation as the ice passes over bumps with plastic flow around bed obstacles increasing the thickness of the debris-bearing clotted ice⁵. The clots were formed by clay and silt aggregating in a sub-glacial environment where liquid water was limited in quantity. The enrichment in heavy isotopes in some of the samples from the dispersed facies is likely to be due to the interaction between hydroxyl-bearing minerals subjected to sub-glacial abrasion and water either at the glacier sole or in the ice where water was in contact with the clots. Subsequent freezing has not obscured this trend in the isotope distribution. The role of the microtome procedure in influencing these results can be excluded as this ice has a low debris content ($<1\%$ by weight). Support for this interpretation comes from our previous results in West Greenland. For example, in the Jakobshavn area a freezing slope is formed by the data on a δD - $\delta^{18}\text{O}$ diagram for basal ice from the stratified facies³—this is caused by the debris being sandy and devoid of fine particles resulting from glacial abrasion. By contrast, in

basal ice from the dispersed facies described above, the presence of clay and silt aggregates with a substantial amount of hydroxyl-bearing minerals has a profound influence on the isotopic characteristics.

For a better understanding of the isotopic characteristics of basal ice from the dispersed facies in West Greenland, the interaction between hydroxyl-bearing minerals and water must be considered. A limited amount of water with very negative δ -values derived from Pleistocene ice will be enriched in heavy isotopes if it comes into contact with hydroxyl-bearing minerals that have been subjected to grinding by sub-glacial abrasion. This is likely to influence the isotopic characteristics of the ice. A co-isotopic study of the ice, both in δD and $\delta^{18}\text{O}$, can show if such an effect has occurred. □

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The timing of crustal extension and the eruption of continental flood basalts

Peter R. Hooper

Department of Geology, Washington State University, Pullman, Washington 99164, USA

THE association between mantle plumes, continental flood basalt eruption and the extension and rifting of the crust is well recognized^{1–3}, but there has always been controversy as to whether the plume or the rifting was the initiating factor^{4,5}. Was rifting triggered by doming above the thermal plume^{2,3,6}, or was rifting the driving force, preceding the associated volcanicity^{7,8}? Here I show that recent flow-by-flow mapping of two continental flood basalt provinces, the Columbia River Basalt Group and the Deccan Basalt Group, allows one to infer the relative timing of volcanicity and crustal extension. In both provinces, eruption of the main tholeiitic phase preceded significant extension and crustal thinning. This suggests that the primary event in the formation of these provinces was the upwelling of a mantle plume, and that the subsequent crustal extension, rifting and decompression was in part a consequence of the initiation of that plume.

Increasingly detailed knowledge of continental flood basalt provinces has highlighted their extraordinary volume and rate of eruption. The basalts are almost entirely tholeiitic and were erupted in a very short period of time. The volume and rate of extrusion of individual eruptive events were also exceptional and were necessary for the sheet form of the flows^{9,10}. On the Columbia Plateau 175,000 km³ of lava accumulated in <2 Myr and within five polarity epochs¹¹. On the Deccan 1×10^6 – 2×10^6 km³ of lava accumulated in <1 Myr^{12–14}, mostly during a single magnetic-polarity epoch^{12–14}. Individual eruptions of 700 km³ have long been recognized on the Columbia Plateau^{9,10}

and volumes $>2,000$ km³ have recently been suggested¹¹. Mapping of individual flows is less advanced on the Deccan, but conspicuous flows have been traced for many tens of kilometres¹² and most of the formations have been traced for hundreds of kilometres^{17,18}. The large volumes and high eruption rates are the hallmarks of continental flood basalt provinces and distinguish such provinces, by an order of magnitude, from the products of other types of volcanic activity. Continental flood basalt provinces were formed sporadically through the geological record and are associated in space and time with both the initiation of hotspots and the initiation of continental rifting.

The Deccan basaltic pile has been mapped for 450 km along the Western Ghats between Nasik and Goa, in west central India (Fig. 1; ref. 18 and references therein). Eruptions started at the north end of the Western Ghats, northeast of Bombay, with the formation of a huge volcanic shield. Younger eruptions form a volcanic ridge running south from the shield on which the individual flows have a southerly dip¹². The implied southward migration of the volcanic centre probably reflects the rapid northward migration of the Indian Plate (at 15 cm yr⁻¹, the plume would appear 150 km further south in 1 Myr). Earlier suggestions that the centre of eruption lay offshore, which are frequently repeated in the literature, seem to be based on the presence of a gravity anomaly northwest of Bombay^{19,20} (Fig. 1). But the strong north-south elongation of the anomaly and the apparent decrease in basalt thickness offshore²¹ make it much more probable that this gravity high is associated with compositionally diverse eruptions that were aligned parallel to the west coast and specifically accompanied later crustal extension in this region²⁰.

The 3,000-m-thick composite section along the Western Ghats is entirely tholeiitic; there are no intermediate, silicic or alkalic rocks present. Down the length of the Western Ghats the stratigraphic sequence is unbroken by any major structural discontinuity. The entire sequence has reversed magnetic polarity, with the exception of a small group of normal flows at the top and a possible normal magnetic-polarity epoch recorded in the

lowest flows of the structurally complex and as yet stratigraphically uncertain flow sequence in the Narmada rift zone²². The whole stratigraphic sequence of the Western Ghats has been dated by $^{40}\text{Ar}/^{39}\text{Ar}$ techniques at 66 ± 1 Myr. The total duration of the mapped eruptive sequence is less than the discriminating ability of the dating technique^{15,23}.

Large dykes, with compositions that can be correlated closely with those of the overlying flows, are exposed in the coastal plain below and west of the Ghats, northeast of Bombay (Fig. 1). The dykes decrease in number upsection, both vertically and toward the south as the southerly dipping flow stratigraphy is crossed. Unlike the highly orientated dyke swarms both to the north (Narmada) and the west, the composition of which are often unrelated to the main tholeiitic eruption, the dykes northeast of Bombay are obvious candidates as feeders for the flows of the main tholeiitic eruption. The orientation of these large probable feeder dykes is essentially random (Fig. 1). This implies that the crust was not under extensional strain when the main phase of the Deccan continental flood basalt province was erupted.

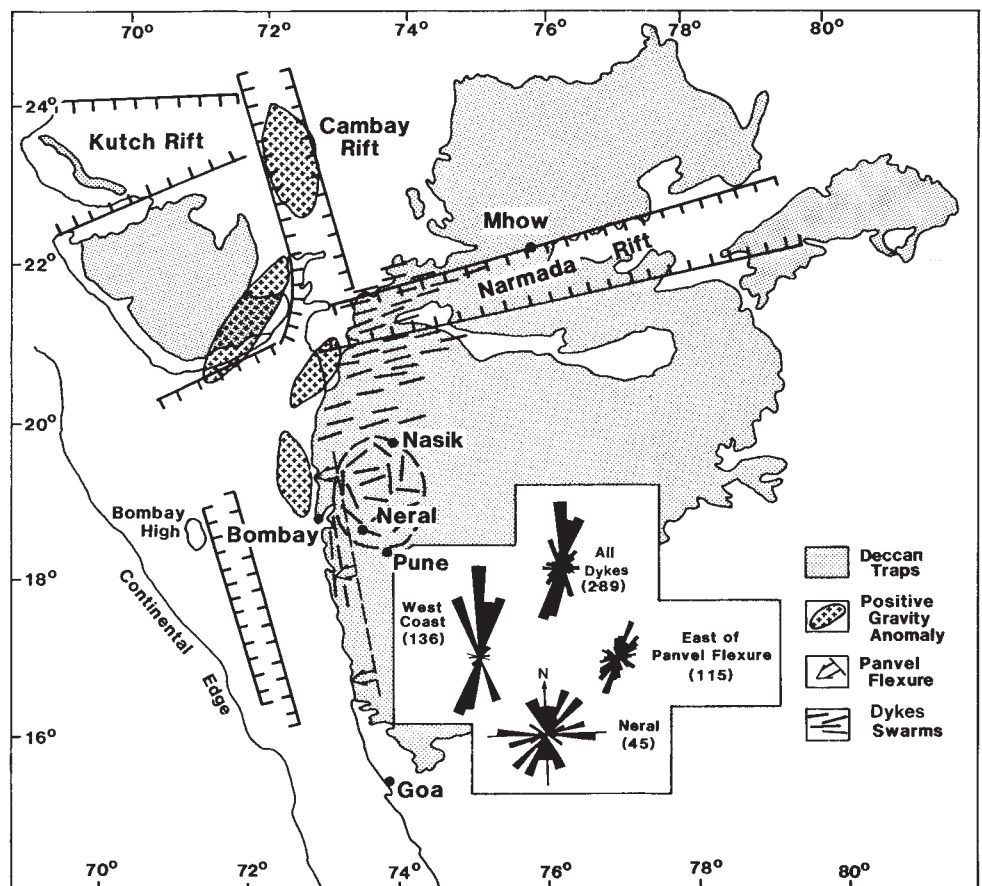
A distinctive dyke suite trends north to north-northwest, parallel to the west coast (Fig. 1). These dykes cut flows of the uppermost (Wai) subgroup downdropped from the top of the Ghats further east by the Panvel Flexure^{24,18} (Fig. 1) and by NNW-trending normal faults. These dykes are, therefore, younger than most of the flows of the main tholeiitic eruptions and cannot represent the feeders to those flows. They are both tholeiitic and lamprophyric in composition. Some may have acted as feeders for the small volumes of intermediate to alkalic flows that lie above the normal Deccan tholeiites on Bombay Island²⁵, which have been dated by Rb/Sr techniques at 64 Myr²⁶. Further west, offshore oil and gas drilling has revealed a series of north to north-northwest trending horst-graben structures where the basaltic pile thins²¹ (Fig. 1). These highly orientated dykes and structures reflect a period of east-west extension

that is younger than the main eruptions of flood basalt and which corresponds in age to the first oceanic crust to form between the Indian and Seychelles Plates²⁷.

Numerous dykes, of both tholeiitic and alkalic compositions, parallel the Narmada fault system (ENE-WSW) north of Nasik (Fig. 1). No systematic study of these dykes has been made, but the presence of alkalic compositions which are conspicuously absent from the main basalt pile of the Western Ghats, and their much wider span of ages, suggests that these dykes are more specifically related to the Narmada structure itself. The Narmada structure has a long and complex history associated with igneous activity older, contemporaneous with, and younger than, the main Deccan eruptions. The present scanty evidence renders it unlikely that the Narmada dykes acted as the primary feeders to the main tholeiitic eruption which reaches its maximum thickness further south.

The Deccan Province lies at the northern end of a long volcanic track, the southern end of which now corresponds to the Réunion hotspot, and has long been thought to represent the initiation of that hotspot¹⁵. Evidence discussed above suggests that the mantle plume produced voluminous melt beneath a thick, fast-moving continental plate which was not under significant extensional strain. The subsequent extension along the west coast of India seems to be, in part, a consequence of the new plume which generated its own type of volcanicity. The modest volume of typically intermediate magma with a strong alkalic component differs in physical style and composition from the initial voluminous plume-related tholeiitic activity. This extension-related volcanicity has much in common with volcanicity associated with extension and thinning of continental crust in provinces such as the Basin and Range and the Rio Grande of western North America. The east-west extension eventually led to the separation of the continental plate into Indian and Seychelles Plates and to the eruption of oceanic tholeiites.

FIG. 1 Sketch map of the west coast of central India, illustrating some of the main structural features (modified from ref. 42) associated with the Deccan Basalt Province. Orientation of dykes in the three principal swarms is shown diagrammatically. Inset displays recorded orientations of dykes in different areas.



Like the Deccan, the Columbia River Basalts of the north-western United States are entirely tholeiitic in character²⁸. The total volume of the Columbia River Basalt Group (CRBG) is much less than the estimated original volumes of the Deccan, the Parana or the Karoo provinces, but the CRBG has the same sheet-flow structure which implies the same extraordinarily rapid rate of eruption during single extrusive events and the same very rapid accumulation of tholeiitic lava on the surface. Some 99% of the CRBG volume was erupted in <2 Myr. These rapid eruption rates of homogeneous evolved tholeiite²⁸ may be regarded as the essential features of a continental flood basalt province.

During the main eruption of the CRBG between 17 and 15 Myr, the Columbia Plateau was subject to mild east-west extensional strain, representing the northern limit of the Basin and Range Province. The degree of extension was <1% and it was accompanied by mild north-south compression, without evidence of crustal thinning^{29,30}. This mild strain produced highly orientated north-south feeder dykes, east-west folds broken by brittle reverse faults, and a conjugate set of NW-trending right-lateral and NE-trending left-lateral strike-slip faults²⁹. The conclusion that this mild strain was not directly connected to the tholeiitic flood eruption derives from the observation that much greater extension, occurring further south in the centre of the Basin and Range Province, was accompanied by only modest volcanicity involving many small eruptions of diverse composition³¹ (see, however, refs 32 and 33).

At ~15 Myr, following the voluminous Grande Ronde Basalt eruptions, the Columbia Plateau was disrupted by right-lateral displacement along the west-northwest orientated Olympic-

Wallowa Lineament³⁰ (OWL; Fig. 2). The strain ellipse represented by the OWL megashear zone and associated structures has the same Basin-and-Range-related orientation as is seen across the rest of the Columbia Plateau³⁰. The OWL separates an area to the south that underwent extension of ~20% from an area to the north in which the amount of extension remained <1%. The 20% extension created a series of north-south grabens and pull-apart structures³⁰ (Fig. 2), the formation of which was accompanied by an abrupt change in the style and composition of the associated volcanicity. In place of the massive tholeiitic sheet-flows (which continued to erupt through the relatively unstrained crust to the north of OWL), the stretching and thinning of the crust on the south side was accompanied by the eruption of small volumes of olivine tholeiites, calc-alkalic to alkalic and silicic volcanics (the Powder River and Weiser volcanic fields)^{34,35}, very similar in their type and diversity to volcanicity associated with other parts of the Basin and Range Province farther south.

Significant extension and crustal thinning on the Columbia Plateau followed, rather than preceded, the eruption of the typical continental flood basalts, as it did on the Deccan. Back-arc spreading associated with the Basin and Range Province is not adequate to explain the massive and very focused eruption of tholeiitic basalt that constitutes the CRBG and which differs so plainly from the distinctive volcanicity associated with Basin and Range crustal extension. The northward jump in the Basin and Range extension (from the Brothers and Vale fault zones to the OWL) seems to have been triggered by a weakening of the crust in the southeastern Columbia Plateau as a result of the massive outpouring of CRBG tholeiite. When significant extension and crustal thinning did occur, it was accompanied by a type of volcanicity contrasting with that of the CRBG in the small volume of individual eruptions, the restriction of the lavas to the grabens, and the diversity of magma composition (which ranges from tholeiitic to silicic, through intermediate calc-alkalic types, and which was always accompanied by an alkalic component). This is the same volcanic assemblage typical of other parts of the Basin and Range Province and of many other provinces associated directly with the extension and thinning of continental crust^{31,36}.

In a series of recent articles, McKenzie and White and their colleagues³⁷⁻³⁹ have developed an elegant model in which crustal extension and the accompanying decompression is the driving force for volcanic eruptions. That volcanic activity can be, and is, caused by such a mechanism is not questioned here, but the implication that continental flood basalts are simply the consequence of decompression caused by crustal extension when a rift system coincides with an established mantle plume seems to be inadequate. More significant is the association of continental flood basalt eruptions both with the initiation of major mantle plumes and with the initiation of crustal rifting. As it seems inconceivable that rifting of the lithosphere can initiate a hot spot the origin of which must lie deep in the mantle, it is probable that mantle plumes are the primary cause of continental flood basalts⁶ and that the new plumes then act as foci for continental splitting in a lithosphere already under regional extensional strain^{3,40}. Timing of crustal extension and eruption of continental flood basalts in the two provinces where these relations are best constrained is consistent with this conclusion. Evidence to determine these relations in the Karoo, Parana and other continental flood basalt provinces is not yet available.

Once started, extension and thinning of continental crust is associated with a type of volcanicity that is very different from that of continental flood basalt provinces; continental extension is associated with eruptions that are of relatively small volume and of diverse composition, typically intermediate to alkaline in character. The degree of alkalinity varies considerably from one extensional province to another and is not well understood; one may speculate that crustal age and thickness are important factors. This assemblage is typified by the Basin and Range and

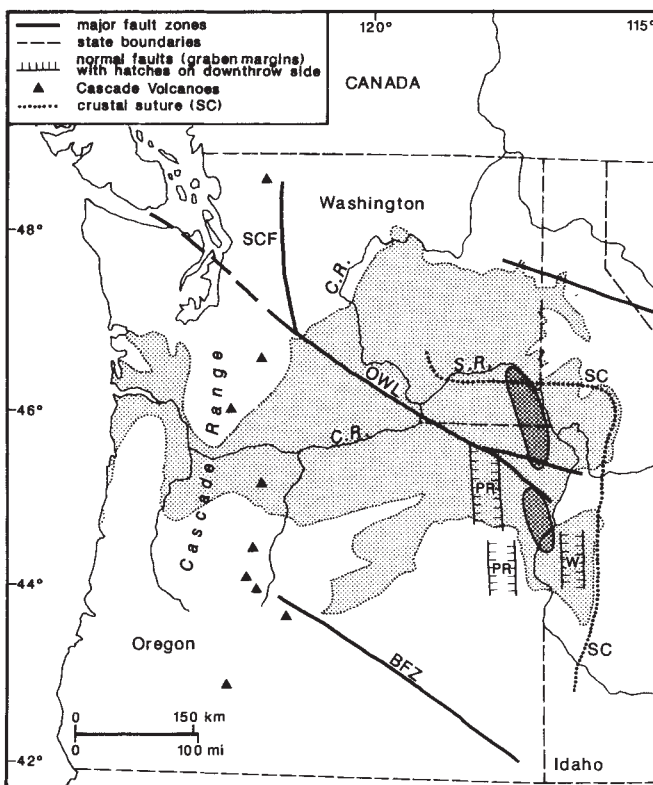


FIG. 2 Sketch map showing the areal extent of the Columbia River Basalts (pale shade), zones of greatest CRBG feeder dyke concentration (dark shade) and related volcanic rocks with pertinent major structures. SCF, Straight Creek Fault; OWL, Olympic-Wallowa Lineament; BFZ, Brothers Fault Zone; PR, Powder River Volcanic Field; W, Weiser Volcanic Field; C. R., Columbia River; S. R., Snake River. The grabens south of the OWL developed after the eruption of the Grande Ronde Basalt (~14.5 Myr ago; D. G. Bailey, personal communication) and their formation was accompanied by eruption of the Powder River and Weiser Volcanic rocks^{30,34,35}. For greater structural detail see ref. 30.

Rio Grande Provinces in western North America, but is represented worldwide where the association between continental rifting and alkalic volcanicity has been long recognized by petrologists⁴¹. □

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Tetraceratops is the oldest known therapsid

Michel Laurin & Robert R. Reisz

Department of Zoology, Erindale Campus, University of Toronto, Mississauga, Ontario, L5L 1C6, Canada

AN unusually rich fossil record documents in great detail the evolution of synapsids (mammal-like reptiles and their descendants the mammals). This fossil record extends over 320 million years and provides extensive evidence of the transition between primitive amniotes and mammals. The superior quality of the synapsid fossil record has even enabled scientists to use it to illustrate the concept of evolution and to test evolutionary models^{1,2}. But a major morphological gap still exists in this fossil record, between Lower Permian and Upper Permian synapsids³. Historically, this gap has been used to divide synapsids into pelycosaurs (primitive mammal-like reptiles) and the geologically younger, more advanced therapsids. We studied *Tetraceratops* to document the origin of therapsids; preparation and restudy of *Tetraceratops insignis*, originally described as a pelycosaur^{4,5} demonstrate that this Lower Permian genus bridges the gap between pelycosaurs and therapsids.

Tetraceratops was collected and described at the beginning of this century⁴. Its evolutionary significance was not recognized because little was known about its osteology. The holotype and only known specimen of this strange reptile had not been prepared thoroughly and restudied in detail⁵, because the surrounding rock was very difficult to remove. *Tetraceratops* is the oldest known horned tetrapod; at least three pairs of horns must have been present on this animal, one on each premaxilla, prefrontal, and angular (the generic name was chosen because only four horn-bearing processes were exposed when Matthew erected this taxon). They confer on this genus a superficial similarity to the primitive therapsid *Burnetia mirabilis*⁶, but this probably does not indicate close relationships between these two synapsids, because the horns are not located on the same bones in both genera. A diastema reminiscent of the dicynodont condition (but probably not homologous to it) interrupts the upper tooth row between the upper incisors and the canine. The bone is extremely thick along the edges of the lateral temporal fenestra, the alveolar shelf, and around the horns. This, along with the exceptionally large size of the first incisor and canine, gives the skull a very peculiar and robust aspect.

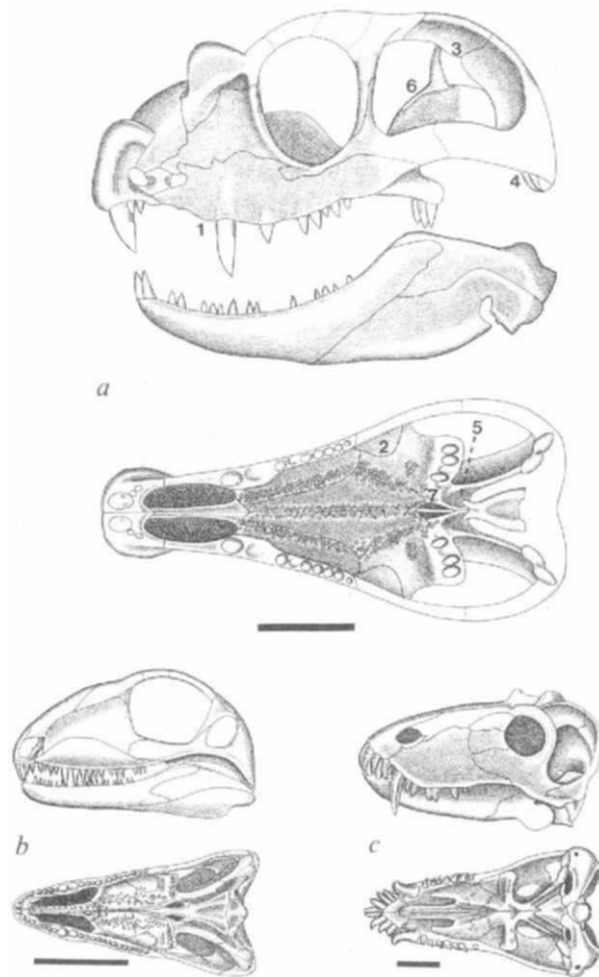


FIG. 1 Reconstructions in left lateral view (above), and in palatal view (below) of (a) *Tetraceratops insignis*, based on the holotype (American Museum specimen number 4526, found in the basal strata of the Clear Fork group of the Big Wichita River, Baylor County, Texas); (b) *Haptodus baylei*, an advanced pelycosaur, redrawn from Currie⁸; and (c) *Titanophoneus*, a primitive therapsid, redrawn from Kemp³. Scale bars, 2 cm. Unshaded areas represent unpreserved, or poorly preserved, parts of the specimen. The numbers refer to the synapomorphies discussed in the text.

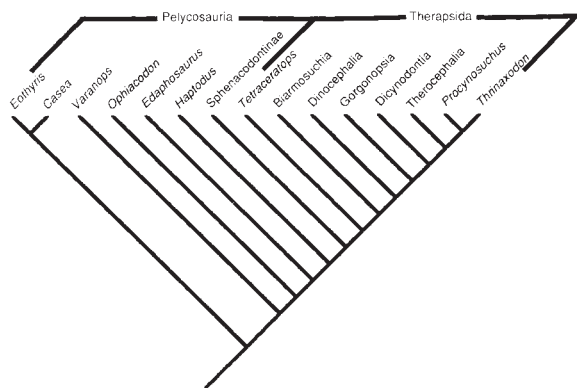


FIG. 2 Phylogeny of synapsids, showing the intermediate position of *Tetraceratops*. This tree results from an analysis of 12 taxa (all the taxa shown except for *Eothyris*, *Casea*, and *Varanops*) and 111 characters. The data matrix was analysed by the branch-and-bound algorithms of PHYLIP 3.1 (ref. 9) and of PAUP 3.0 (D. L. Swofford, PAUP version 3.0, instruction manual) that guarantee to find all the most parsimonious trees. Only one tree was found. The relationships of *Eothyris*, *Casea*, and *Varanops* to other pelycosaurs had been previously established by one of us⁷. It had previously been suggested that *Haptodus*⁸ was the closest relative of therapsids. Our results show that *Tetraceratops* is more closely related to all previously known therapsids than any pelycosaur, and that sphenacodontines are more closely related to therapsids than *Haptodus*.

Tetraceratops and all previously known therapsids form a monophyletic group excluding sphenacodontines (the closest pelycosaurian relatives of therapsids; they include *Dimetrodon*, *Sphenacodon*, *Ctenospondylus*, and a few other poorly known genera⁷) because they share the following seven derived features which appear in the primitive condition in sphenacodontines: (1) the precanine teeth are lost; (2) the ectopterygoid teeth are lost; (3) the upper margin of the lateral temporal fenestra is broadened and forms a new concave, ventro-laterally facing surface from which the jaw adductor musculature originated; (4) the quadrate, the upper element of the jaw suspension, is reduced in size; (5) a posteromedian flange of the pterygoid is present behind the interpterygoid vacuity; (6) the ventral plate of the epipterygoid is much reduced in size; (7) the interpterygoid vacuity is reduced in length. (See Fig. 1 for illustration of these points.)

The presence of a number of diagnostic therapsid features cannot be tested in *Tetraceratops* because of the fragmentary nature of the specimen. Nevertheless, all Upper Permian and later therapsids form a monophyletic group excluding *Tetraceratops* because they share the following derived characters that appear in the primitive state in *Tetraceratops* and pelycosaurs: the septomaxilla is large and has a facial process that excludes the maxilla from the narial margin; the maxilla contacts the prefrontal; the maxilla contacts the nasal and excludes the lacrimal from the naris; a distinct incisiform region is present; all the incisors are equal in size; the basiptyergoid articulation is absent.

This combination of primitive and advanced features leaves little doubt that *Tetraceratops* is intermediate between pelycosaurs and all previously known therapsids. However, the seven derived characters shared with therapsids imply that *Tetraceratops* must be placed on the therapsid side of the dichotomy between the pre-existing sister-taxa Sphenacodontinae and Therapsida.

Pelycosaurs first appear in the fossil record in the Middle Pennsylvanian, and disappear in the Late Permian, reaching their greatest diversity in the Early Permian. Therapsids, their descendants, were previously known from the Late Permian through the Jurassic. It is therefore not totally unexpected that

Tetraceratops, the oldest known therapsid, comes from the upper strata of the Early Permian of Texas; the great diversity of therapsids in the lowest strata of the Upper Permian of the Soviet Union and South Africa suggests that they arose much earlier.

As the most primitive known therapsid, *Tetraceratops* bridges the large morphological gap between primitive and advanced mammal-like reptiles. This makes the fossil record of synapsids the most extensive of any group of terrestrial vertebrates, extending without any significant break through the last 320 million years of Earth's history. The recognition of *Tetraceratops* as the oldest and most primitive known therapsid may also help to resolve the problem of the origin of therapsids. □

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Regulated expression and binding of three VLA (β 1) integrin receptors on T cells

Yoji Shimizu, Gijis A. Van Seventer, Kevin J. Horgan & Stephen Shaw

Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA

REGULATED adhesion of T cells to extracellular matrix (ECM) proteins is likely to be essential in T cell migration. Constitutive binding of various other cell types to ECM components is mediated by members of the VLA (very late antigen) subfamily of integrins^{1–4}. We describe here the regulated binding of resting CD4⁺ human T cells to ECM through three VLA integrins: VLA-4 (refs 5, 6) and VLA-5 (ref. 7) binding to fibronectin (FN), and a novel pathway of VLA-6 binding to laminin (LN). Binding to ECM is regulated in two ways. First, unlike other VLA-mediated interactions, VLA binding activity of the T cells is rapidly and dramatically augmented with cell activation without change in level of expression of the VLA molecules. Second, binding is regulated with T-cell differentiation; memory T cells express three- to four-fold more VLA-4, VLA-5, and VLA-6 than do naive cells, and bind more efficiently through them to FN and LN.

To address the possible importance of VLA molecules in T-cell migration, we investigated: (1) expression of VLA molecules on CD4⁺ resting human T cells; and (2) *in vitro* interactions of CD4⁺ T cells with purified ECM proteins known to be ligands for integrins. Despite the name 'very late antigen', VLA-4, VLA-5, and VLA-6 α chains and their common β 1 chain are expressed on CD4⁺ T cells (Fig. 1).

Studies such as those displayed in Fig. 2 show that strong T-cell binding is observed both to laminin (LN) and fibronectin (FN) but only after activation of the T cell. Monoclonal antibody (mAb) blocking studies show that T-cell binding to both LN and FN is mediated by VLA integrins, as it is completely inhibited by a VLA- β 1 chain-specific mAb but not by mAb specific for the β 2 integrin subfamily, which are also expressed

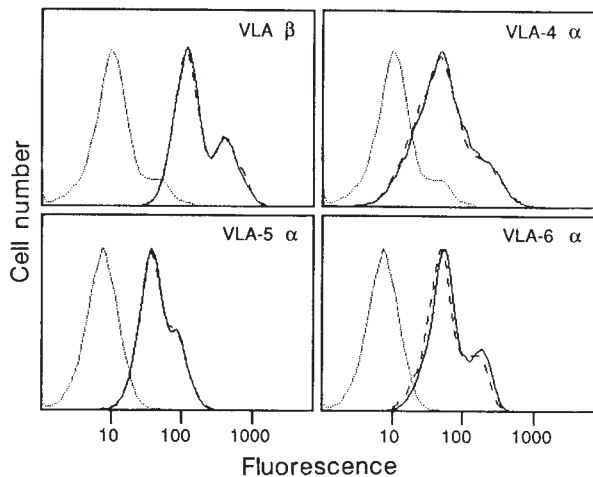


FIG. 1 Expression of VLA integrins on resting peripheral human CD4⁺ T cells. Typical fluorescence histograms for negative control mAb (.....) or VLA β 1 mAb, VLA-4 mAb, VLA-5 mAb, VLA-6 mAb binding to resting CD4⁺ T cells (—) or CD4⁺ T cells acutely activated with TPA (---).

METHODS. Purified CD4⁺ T cells were prepared by exhaustive negative selection from peripheral blood mononuclear leukocytes of normal donors using immunomagnetic beads³⁰; purity of the CD4⁺ cells was greater than 98%. Since the selection cocktail of mAb included the anti-HLA-DR mAb IVA12, cells had been preselected to exclude the normal low percentage of circulating activated T cells. Staining and flow cytometric analysis were carried out by standard procedures³¹ using a FACScan (Becton-Dickinson). MAbs used were 4B4 (VLA β 1, Coulter Electronics), L25 (refs 23, 32) (VLA-4, Dr E. Evans, Mountain View), BIIG2 (ref. 33) (VLA-5, Dr C. Damsky, San Francisco), and GoH-3 (ref. 15) (VLA-6, Dr A. Sonnenberg, Amsterdam). A goat anti-mouse IgG sandwich was used for the mouse mAbs 4B4 and L25 and goat anti-rat IgG (H+L) was used for the rat mAbs BIIG2 and GoH-3. T cells were activated by a 10 min incubation at 37 °C with 10 ng ml⁻¹ TPA, diluted into ice-cold media, and stained as described above. Results were similar when the duration of TPA incubation was 60 min (not shown).

on human T cells (Fig. 3a). Blocking studies with VLA- α chain-specific mAb (for T cells activated by either 12-O-tetradecanoylphorbol-13-acetate (TPA) or CD3 mAb cross-linking) (Fig. 3b) show that: (1) VLA-6 mediates binding to LN; and (2) VLA-4 and VLA-5 both mediate binding to FN. Although most FN binding is mediated by VLA-5, a contribution by VLA-4 is shown by the capacity of mixes of VLA-4 and VLA-5 mAb to inhibit binding to FN completely.

T-cell adhesion to FN and LN is minimal unless the T cells are activated (Fig. 2). Binding is induced within 10 min by activation of the cells by way of two physiologically important cell surface receptors: the CD3/T-cell receptor complex⁸ and the CD2 molecule⁹ (Fig. 2). TPA induces similar strong binding. The level of expression of VLA-4, VLA-5, and VLA-6 is not increased during this induction (Fig. 1). Thus, activation dramatically augments VLA-mediated T-cell binding without increasing VLA expression.

The bimodal expression of VLA β 1 on CD4⁺ T cells (Fig. 1) distinguishes two functionally distinct reciprocal subsets¹⁰ which are now interpreted to be two stages of T-cell differentiation: 'naive' and 'memory' cells. Naive cells emerge from the thymus and become memory cells after stimulation by specific antigen^{11,12}. We compared the expression of individual VLA molecules on naive and memory cells distinguished by their

differential expression of CD45RA, the most widely used discriminator of these subsets. Whereas VLA-1 and VLA-2 are not expressed on resting CD4⁺ T cells¹, and VLA-3 is expressed at most minimally, the β 1 chain and the VLA-4, VLA-5 and VLA-6 α chains are each expressed about 3- to 4-fold higher on memory cells than on naive cells (Fig. 4a).

The higher expression of VLA-4, VLA-5, and VLA-6 on memory T cells is associated with markedly greater binding of purified memory cells to LN and FN than purified naive cells from the same donor (Fig. 4b). Like the CD4⁺ unseparated cells, binding of each subset depends on activation (through CD3 or CD2, for example). mAb blocking studies indicate that binding mediated by each of the VLA-4, VLA-5, and VLA-6 molecules is augmented in memory cells (not shown).

The regulated interaction of T cells with ECM through VLA integrins is likely to be critical to lymphocyte migration. The novel finding that T cells express VLA-6 and use it to bind to LN may be critical to T-cell binding and penetration through basement membranes, where LN is characteristically found^{13,14}; VLA-6-mediated binding of platelets to LN has also recently been described¹⁵. VLA-4- and VLA-5-mediated T-cell binding to FN (refs 5-7) should allow T cells to interact with interstitial matrix, where FN is prevalent. Furthermore, these three pathways may also facilitate cell-cell binding, as: (1) surface LN

FIG. 2 Binding of CD4⁺ T cells to fibronectin (FN) and laminin (LN) can be induced by acute cell activation. Binding of ⁵¹Cr-labelled CD4⁺ T cells to LN (left panel) or FN (right panel) after no activation (□) or after activation for 10 min at 37 °C with 10 ng ml⁻¹ TPA (●), the CD3 mAb OKT3 crosslinked by goat anti-mouse immunoglobulin (■) or a combination of the CD2 mAbs 95-5-49 (10 μ g ml⁻¹) and 9-1 (1:2000) (▲). To the right of the dotted line in each panel is the binding of resting and activated T cells to type I collagen applied at a concentration of 1 μ g per microtitre well.

METHODS. Resting human CD4⁺ T cells were isolated as described in the legend to Fig. 1. Human FN, human LN (both Telios Pharmaceuticals, San Diego), and human type I collagen (Sigma) were applied to 96-well flat bottom microtitre wells (Costar) at the concentrations indicated in PBS medium at 4 °C overnight; unbound binding sites on plastic were blocked with PBS/2.5% BSA for 2-3 h at 37 °C. ⁵¹Cr-labelled T cells (50,000 per well) were added in a final volume of 0.1 ml PBS/0.5% human serum albumin (HSA). For the PMA and CD2 activation conditions, cells were added to wells containing the indicated amounts of PMA and CD2 mAb pair. For CD3 activation, T cells were incubated with 10 μ g ml⁻¹ of OKT3 mAb for 30 min at 4 °C, unbound mAb was removed by two washes at 4 °C with PBS/HSA and added to wells containing 0.5 μ g ml⁻¹ goat anti-mouse Ig (Organon). After 1 h settling at 4 °C, plates were rapidly warmed to 37 °C for 10 min, non-adherent cells washed off, and the percentage of bound cells determined by lysing with detergent, and counting gamma emissions from the well contents. Data are expressed as the mean per cent of cells binding from three replicate wells.

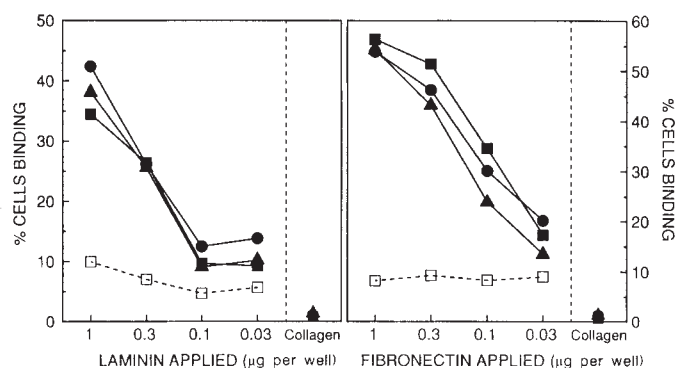
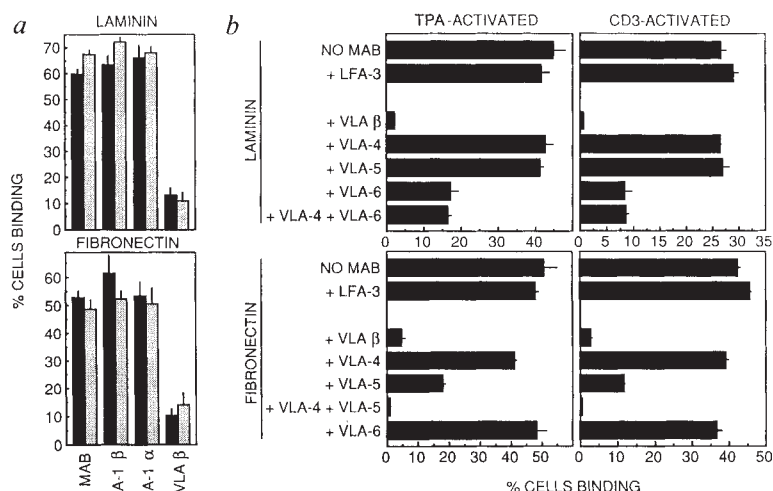


FIG. 3 Activation-dependent binding of CD4+ human T cells is mediated by three VLA integrins. *a*, Binding of TPA-activated (solid bars) and CD3-activated (shaded bars) CD4+ T cells to LN (top panel) and FN (bottom panel) in the presence of mAb specific for the VLA β 1 chain and the α and β chains of the related LFA-1 β 2 integrin. *b*, Determination of ligand specificity of VLA-4, VLA-5 and VLA-6 by mAb blocking of the binding of PMA-activated (left panels) and CD3-activated (right panels) CD4+ T cells to LN (top panels) and FN (bottom panels).

METHODS. Binding assays were carried out as described in the legend to Fig. 2. CD3 activation used the IgM CD3 mAb 38.1 (Dr P. Martin, Seattle) and goat anti-mouse IgM (Dr G. Laszlo, Bethesda) to prevent binding of blocking mAb by the goat anti-mouse immunoglobulin. Binding to LN and FN (both applied at 1 μ g per microtitre well) was assessed in the presence of the following blocking mAb: the LFA-1 β chain mAb MHM23, the LFA-1 α chain mAb MHM24, the negative control LFA-3 mAb TS2/9, the VLA β 1 mAb 4B4, the VLA-4 mAb L25, the VLA-5 mAb BIIG2, and the VLA-6 mAb GoH-3. MHM23, MHM24, TS2/9, 4B4, and L25 were used as purified mAb at 10 μ g ml⁻¹, BIIG2 at 1:1000 dilution of ascites fluid, and GoH-3 at 1:20 dilution of culture supernatant. Data from two separate experiments with different donors are presented in (a) and (b). Binding of resting T cells in (a) to FN was 15% and to LN was 17%; in (b), binding of resting T cells to FN was 8% and to LN was 5%. Binding of activated cells from both donors to an irrelevant ECM protein, type I collagen, was <3%, and has not been subtracted from the data shown.



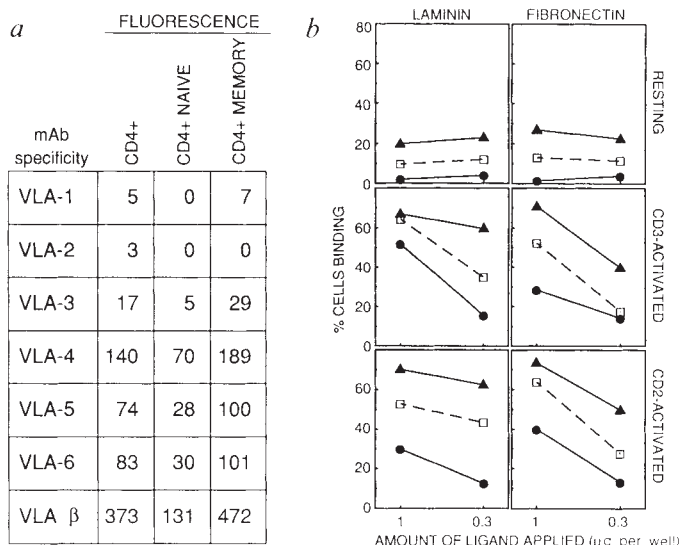
and FN have been observed on various cells including some tumour cells^{16,17}; and (2) various T-cell responses that are dependent on cell-cell interactions have been reported to be affected by ECM proteins (refs 18–21 and Y.S. *et al.*, submitted) or VLA mAb (refs 21–24 and Y.S. *et al.*, submitted).

Activation-regulated function is unprecedented for VLA integrins, which are constitutively capable of binding in all other model systems studied. But rapid induction of adhesive capacity has been demonstrated for the β 2 integrin LFA-1 on T cells²⁵ and for β 2 and β 3 integrins on other cell types²⁶. This rapid

induction of VLA binding capacity upon activation may enable T cells to persist at sites where they encounter cells that activate them – for example, sites of tissue inflammation or in lymphoid organs such as lymph node or thymus²⁷. A second mode of regulation of VLA integrin binding is the coordinate increased expression of VLA-4, VLA-5 and VLA-6 integrins as a result of T-cell differentiation from naive to memory cells. This augmented adhesion may contribute to enhanced migration into tissue and thereby account, at least in part, for preferential localization of memory cells in tissue, at mucosal surfaces, and

FIG. 4 Expression and function of VLA integrins on CD4+ naive and memory T cells. *a*, Quantitation of binding of VLA mAbs to CD4+ T cells or subsets of these cells from a representative donor. *b*, Binding of purified naive and memory human CD4+ T cells to LN and FN after acute CD3- and CD2-mediated activation. Binding of resting (top panels), CD3-activated (middle panels), and CD2-activated (bottom panels) CD4+CD45RA+ naive T cells (●), and CD4+CD45RA– memory T cells (▲) to LN (left panels) and FN (right panels) was assayed. Binding of the unseparated CD4+ T-cell preparation from the same donor is also shown (□).

METHODS. *a*, Purified CD4+ T cells were prepared as described in the legend to Fig. 1. Staining and flow cytometric analysis were performed as described³¹ using sequential incubations with: (1) saturating concentrations of test mAb; (2) Texas Red-labelled anti-immunoglobulin sandwich; (3) excess irrelevant IgG to block free sites on bound sandwich; (4) CD45RA mAb Leu-18-FITC (Becton-Dickinson). Logarithmic amplification was provided by a three-decade logarithmic amplifier and the data for median fluorescence converted to arbitrary linear units. Data shown are after subtraction of background fluorescence of cells in the presence of an irrelevant mAb. MAb used were 4B4 (VLA β 1), VLA-1 (T Cell Sciences, Cambridge, MA), Thr/4 (VLA-2, Dr A. von dem Borne, Amsterdam), J143 (ref. 34) (VLA-3, Dr L. Old, New York), L25 (refs 23, 32) (VLA-4), BIIG2 (ref. 33) (VLA-5), and GoH-3 (ref. 15) (VLA-6). Discrimination between naive and memory cells was by electronic discrimination of the two peaks of CD45RA expression. *b*, Purified CD4+ CD45RA+ ('naive') and CD4+ CD45RA– ('memory') T cells from the same donor were prepared by exhaustive negative selection as described³⁰. Purity of naive and memory subsets was greater than 95% as determined by flow cytometric analysis. Adhesion assays and activation conditions were carried out as described in the legend to Fig. 2. LN and FN were applied at 1 μ g per microtitre well as described in the legend to Fig. 2.



at sites of inflammation^{28,29}. Thus, T cells express an ensemble of VLA molecules capable of interacting with multiple ECM components that may critically influence their migration. □

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Chloroquine resistance not linked to *mdr*-like genes in a *Plasmodium falciparum* cross

Thomas E. Wellems*, Lindsey J. Pantón†, Ilya Y. Gluzman‡, Virgilio E. do Rosario†, Robert W. Gwadz*, Annie Walker-Jonah* & Donald J. Krogstad‡

*Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA

†Departments of Medicine and Pathology, Washington University School of Medicine, St Louis, Missouri 63110, USA

CHLOROQUINE is thought to act against falciparum malaria by accumulating in the acid vesicles of the parasite and interfering with their function^{1–4}. Parasites resistant to chloroquine expel the drug rapidly in an unaltered form, thereby reducing levels of accumulation in the vesicles⁵. The discovery that verapamil partially reverses chloroquine resistance *in vitro*⁶ led to the proposal that efflux may involve an ATP-driven P-glycoprotein pump similar to that in mammalian multidrug-resistant (*mdr*) tumor cell lines. Indeed, *Plasmodium falciparum* contains at least two *mdr*-like genes^{7,8}, one of which has been suggested to confer the chloroquine resistant (CQR) phenotype^{7,9,10}. To determine if either of these

genes is linked to chloroquine resistance, we performed a genetic cross between CQR and chloroquine-susceptible (CQS) clones of *P. falciparum*. Examination of 16 independent recombinant progeny indicated that the rapid efflux phenotype is controlled by a single gene or a closely linked group of genes. But, there was no linkage between the rapid efflux, CQR phenotype and either of the *mdr*-like *P. falciparum* genes or amplification of those genes. These data indicate that the genetic locus governing chloroquine efflux and resistance is independent of the known *mdr*-like genes.

The CQS parent of the cross, clone HB3 from the Honduras isolate Hondo-1¹¹, exhibited full susceptibility to chloroquine (IC₅₀ 8 ng ml⁻¹; Table 1). The resistant parent, clone Dd2 from

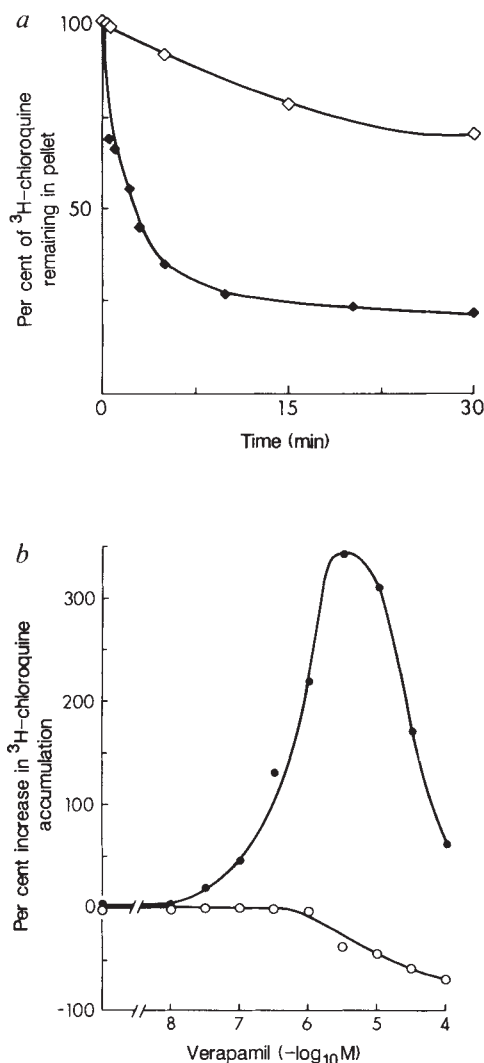


FIG. 1 *a*, Chloroquine efflux curves of the CQS HB3 (◇) and CQR Dd2 (◆) parental clones. *b*, Effect of verapamil on steady-state chloroquine accumulation levels. HB3 (○), Dd2 (●).

METHODS. *a*, Efflux was determined from 1×10^6 parasitized red cells ml⁻¹ suspended in culture medium containing 1 or 10 ng ml⁻¹ (3 or 30 nM) ³H-chloroquine (15.3 Ci mmol⁻¹, NEN). After incubation (1 h), parasites were washed twice in medium containing unlabelled chloroquine and resuspended in culture medium without chloroquine. Parasites were centrifuged through silicon oil at selected time points. Efflux was determined from the radioactivity remaining in cell pellets digested with PROTOSOL (NEN). *b*, Steady state chloroquine accumulation levels were measured by incubating 1×10^6 parasites ml⁻¹ in complete medium containing ³H-chloroquine (1 or 10 ng ml⁻¹) with and without 10 μM verapamil. After 1 h, parasites were pelleted through silicon oil. ³H-chloroquine accumulation was expressed as % change in radioactive counts obtained with versus without 10 μM verapamil.

† Present addresses: Departments of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, USA (L.J.P.) and Genetics Department, Edinburgh University, Edinburgh EH9 3JN, UK (V.E.d.R.).

the W2-MEF line of the Indochina III isolate^{12,13}, was eightfold less responsive to chloroquine with a typical IC_{50} for a resistant strain (IC_{50} 64 ng ml⁻¹). Verapamil reversed the chloroquine-resistance of the Dd2 clone but did not affect the susceptibility of HB3, as described previously for resistant and susceptible parasites⁶. Chloroquine efflux halftimes and drug accumulation levels were likewise consistent with CQR and CQS parasites studied previously (Fig. 1). Efflux of chloroquine from Dd2 parasites was rapid ($t_{1/2}$ 1–2 min) whereas it was much slower ($t_{1/2}$ 60–70 min) from HB3 parasites. Verapamil (10 μ M) increased chloroquine accumulation in the Dd2 parasites by 300%, but actually decreased chloroquine accumulation by 14% in HB3 parasites.

Progeny from the HB3 $\times$ Dd2 cross were generated using a strategy similar to that previously described¹⁴. From 76 progeny clones, 16 clones exhibited unique combinations of parental markers (independent recombinants) with an even distribution of CQS and CQR forms (Table 1). The eight CQS recombinants possessed a chloroquine-susceptible phenotype identical with the HB3 parent. Likewise, the eight CQR recombinants exhibited a phenotype identical with Dd2. No progeny clones demonstrated intermediate phenotypes indicative of multigenic inheritance of chloroquine resistance.

Recent reports have shown that *P. falciparum* contains two genes (*pfmdr1*, *pfmdr2*) homologous to P-glycoprotein genes from mammalian *mdr* tumour cell lines^{7,8}. One of these genes, *pfmdr1*, was found to be amplified in some drug-resistant *P. falciparum* strains. To determine whether either of these genes was linked to chloroquine resistance in the HB3 $\times$ Dd2 cross, we performed polymerase chain reaction (PCR) experiments similar to those previously described⁸. PCR products from *pfmdr1* and *pfmdr2* were isolated, cloned into plasmid vectors, verified by sequencing and used to probe restricted DNA from the HB3 $\times$ Dd2 cross. Sequences from the PCR products were identical with sequences reported from *pfmdr1*⁷ and *pfmdr2*⁸. Figure 2 shows signals from restriction fragments after hybridization with the *pfmdr1* probe. A *Bam*HI polymorphism at the 3' end of *pfmdr1* distinguishes the HB3 (3.2 Kilobases (kb)) and Dd2 genes (3.3 kb). Because asexual, erythrocytic stage parasites are haploid¹⁴, individual progeny exhibit the *pfmdr1* gene of only one parent or the other. The inheritance pattern observed demonstrates that *pfmdr1* is not linked to chloroquine resistance (Fig. 2).

Amplification of the *pfmdr1* gene in the HB3 $\times$ Dd2 cross was assessed from known amounts of genomic DNA¹⁵. Results of this analysis (Table 1) revealed one copy of *pfmdr1* in the HB3 genome and four copies in the Dd2 genome. Among the progeny which had inherited *pfmdr1* from the Dd2 parent, amplified

copies were detected both in CQS and CQR forms. Interestingly, the actual levels of *pfmdr1* amplification varied among progeny having the Dd2 gene. Two clones (3B-B1, SC-01) contained four copies of the Dd2 gene, four clones (B1-SD, 3B-D5, QC-23, TC-05) contained two copies, and one clone (TC-08) contained one copy. By contrast, progeny which had inherited *pfmdr1* from the CQS HB3 parent all exhibited a gene copy number of one (Table 1). Long-range restriction studies have shown that amplification of the *pfmdr1* gene occurs in large, tandemly repeated elements on chromosome 5⁷. One possible explanation for copy-number variation among progeny is that amplified elements were lost with pairing of the HB3 and Dd2 chromosomes during meiosis.

To further test whether *pfmdr1* affected the chloroquine phenotype in the cross, uncloned progeny were taken directly from chimpanzee blood and cultivated *in vitro* with 32 ng ml⁻¹ chloroquine. Under these conditions, growth of resistant progeny was robust while chloroquine susceptible progeny did not survive. Figure 2 shows that no selection of the Dd2 *pfmdr1* restriction fragment length polymorphism (RFLP) occurred in the uncloned progeny cultivated with chloroquine (M1/CqS) compared to progeny cultivated without the drug (M1).

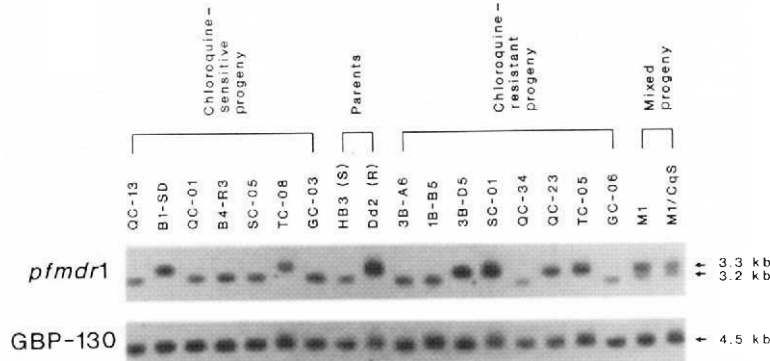
The *pfmdr2* gene was likewise examined for linkage to chloroquine resistance. A search of over 25 restriction enzymes revealed no detectable amplification or polymorphisms of this gene in the HB3 and Dd2 parents. We did, however, identify an RFLP of an anonymous probe (plasmid pSc11.90) which maps near *pfmdr2* (both sequences are found within a 500 kb *Bss*HII/*Bgl*II restriction fragment from chromosome 14). Examination of the inheritance of pSc11.90 among the progeny revealed no linkage to chloroquine resistance (data not shown).

Previous studies of the W2-MEF line, produced by continuous mefloquine pressure to the W2 clone¹², demonstrated amplification of the *pfmdr1* gene and enhanced transcript levels⁸. But, this amplification was not associated with increased chloroquine resistance since the W2-MEF line showed no increase in chloroquine resistance levels over W2^{12,13}. To determine whether mefloquine susceptibility was linked with *pfmdr1* amplification in the HB3 $\times$ Dd2 cross, we examined mefloquine IC_{50} values of the parent and progeny clones. There were no significant differences among the parent and progeny clones, indicating that mefloquine resistance also may not be linked to the *pfmdr1* gene.

Is chloroquine resistance in *P. falciparum* governed by more than one major genetic locus? A gradation of chloroquine resistant forms would have been expected from the HB3 $\times$ Dd2 cross if classic multigenic inheritance were involved. The data in Table 1, however, show a remarkable uniformity among the resistant

FIG. 2 Inheritance of *pfmdr1* in the HB3 $\times$ Dd2 cross. Upper panel shows signals from genomic DNA digested with *Bam*HI and *Eco*RI, and probed with plasmid pPG1-B, containing nucleotides 4,006–4,503 of *pfmdr1*⁷. Signals from the CQS and CQR progeny clones are arranged to the left and right of the HB3 and Dd2 parents, respectively. Far right are results from mixed progeny taken directly from the chimpanzee and cultivated without (M1) and with (M1/CqS) chloroquine. Relative amounts of DNA on the blot were confirmed by a second hybridization with pGBP-6, a clone from the unrelated GBP-130 gene³⁰ on chromosome 10.

METHODS. Forward and reverse PCR primers were generated from regions of *M. musculus mdr*³¹ adapted to *P. falciparum* codon usage³². Amino acid sequences VG₅SGCGKST (*mdr* residues 425–434, 1,067–1,076) and DEATSALD (residues 554–561, 1,198–1,205) were used to derive primers 5'GT_A-GG_A-TC_A-TC_A-GG_A-TG_C-GG_A-AAA-TC_A-AC-3' (forward) and 5'-ATC-TAA- Δ GC- Δ GA- Δ GT- Δ GC-TTC-ATC-3' (reverse). Twenty-seven PCR cycles were performed, each consisting of denaturation for 2 min at 93 °C, renaturation for 2 min at 39 °C and extension for 4 min at 70 °C. PCR



products were isolated by preparative agarose gel electrophoresis and cloned into the *Sma*I site of pBS (Stratagene). pGBP-6 DNA was kindly provided by J. Ravetch. Between hybridizations the blot was stripped with NaOH¹⁵.

TABLE 1 Chloroquine phenotypes and *pfmdr1* inheritance in the HB3 × Dd2 cross

	IC ₅₀ Cq	IC ₅₀ Cq + V	t _{1/2} efflux	Δ _{accum}	<i>pfmdr1</i> RFLP	Copy number
Parent clones						
HB3	8	8	60	-14%	H	1
Dd2	64	16	2	+300%	D	4
Cq-susceptible progeny						
3B-B1	8	8	60	-12%	D	4
QC-13	8	8	60	-24%	H	1
B1-SD	8	8	65	-23%	D	2
QC-01	8	8	60	-16%	H	1
B4-R3	8	8	60	-26%	H	1
SC-05	8	8	60	-26%	H	1
TC-08	6	6	50	-17%	D	1
GC-03	6	6	65	-27%	H	1
Cq-resistant progeny						
3B-A6	64	20	2	+200%	H	1
1B-B5	64	12	1	+230%	H	1
3B-D5	64	16	2	+370%	D	2
SC-01	64	12	2	+290%	D	4
QC-34	64	12	2	+400%	H	1
QC-23	64	12	2	+280%	D	2
TC-05	64	8	1	+170%	D	2
GC-06	64	12	2	+220%	H	1

HB3 and Dd2 gametocytes were produced *in vitro*^{23,24}, mixed and fed to *Anopheles freeborni* mosquitoes²⁵. After 15 days, 300 mosquitoes were collected and fed on a 15 kg splenectomized chimpanzee. More than 90% of the mosquitoes engorged. Patent blood stage infection became evident on day 11. Individual progeny were cloned by the technique of limiting dilution²⁶, either directly from chimpanzee blood or from cultures first established *in vitro* with human red blood cells. Drug susceptibility assays and RFLP analysis (including two unique 'fingerprint' probes to interspersed repetitive sequences and over 30 single-copy RFLP markers to individual chromosomes) were used to group progeny clones according to unique combinations of parental markers. Initial screening identified 14 independent recombinants, with predominance of the 'knobless' phenotype^{27,28} (Dd2 phenotype, KA-HRP²⁹). Uncoloned progeny were therefore cultivated and passed through gelatin²⁹, yielding two additional independent recombinant clones with the knobby phenotype of the HB3 parent (GC-03, GC-06). No parental forms were obtained from the progeny clones. The HB3 clone has been shown previously to be mosquito transmissible¹⁴. Although control experiments with the Dd2 parent revealed the irregular presence of oocysts in *A. freeborni*, transmission to chimpanzees of the Dd2 clone alone was not attempted. Drug susceptibilities were determined by microscopic examination of cultures exposed to serial drug dilutions in microtitre plates. IC₅₀ values represent ng ml⁻¹ chloroquine base at which parasitaemia was reduced by >50% compared to control. Efflux half-times and effect of verapamil were performed as described in Fig. 1. *pfmdr1* inheritance was determined from a *Bam*HI RFLP at the 3' end of the gene (Fig. 2). Copy numbers of the *pfmdr1* gene were determined from Southern blot analysis of known amounts of restricted DNA¹⁵. Cq, chloroquine; V, 0.6 μM verapamil; Δ_{accum}, change in steady-state Cq accumulation with 10 μM verapamil; H and D indicate the HB3 and Dd2 RFLPs of *pfmdr1*, respectively. Efflux half-times are in min.

progeny. Although one cannot yet exclude the possibility that two or more unlinked genes may be required for the CQR phenotype, the simplest explanation is that a single genetic locus governs chloroquine resistance. In the accompanying paper, Foote *et al.*¹⁶ have shown that certain *pfmdr1* mutations are more frequent in chloroquine resistant parasites. The hypothesis that a mutant or competent form of *pfmdr1* is necessary for chloroquine resistance will, however, require perfect association between mutant alleles and the resistant phenotype.

Results from the HB3 × Dd2 *P. falciparum* cross indicate that the rapid efflux, CQR phenotype is independent of the *pfmdr1* and *pfmdr2* genes. The history of the spread of chloroquine resistance suggests that acquisition of resistance involved very rare molecular events in *P. falciparum*. In contrast to pyrimethamine and cycloguanil resistance, which arose independently in many regions from point mutations in dihydrofolate reductase¹⁷⁻²⁰ chloroquine resistance spread slowly during the last 40 years from foci in Southeast Asia and South America. Some evidence indicates that chloroquine resistance in South America may have been imported from a single focus in Southeast Asia^{21,22}. Even if independent foci were involved, all chloroquine-resistant strains so far examined have shown similar efflux phenotypes⁵. These observations are consistent with emergence of a novel mechanism of chloroquine efflux in *P. fal-*

ciparum. Linkage analysis studies are now being performed to search for the genetic locus governing this mechanism. □

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Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*

S. J. Foote, D. E. Kyle*, R. K. Martin*, A. M. J. Oduola*, K. Forsyth, D. J. Kemp and A. F. Cowman

The Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria 3050, Australia

* The Walter Reed Army Institute of Research, Washington DC, USA

THE lethal form of human malaria caused by *Plasmodium falciparum* is virtually uncontrollable in many areas because of the development of drug resistance, in particular chloroquine resistance (CQR). CQR is biologically similar to the multiple drug resistance phenotype (MDR) of mammalian tumour cells, as both involve expulsion of drug from the cell and both can be reversed by calcium channel antagonists¹. A homologue (*pfmdr1*) of the mammalian multidrug resistance gene has been implicated in CQR because it is amplified in some CQR isolates of *P. falciparum*^{2,3} as is an *mdr* gene in MDR tumour cells⁴. We show here that the complete sequences of *pfmdr1* genes from 2 CQ sensitive (CQS) *P. falciparum* isolates are identical. In 5 CQR isolates, 1-4 key nucleotide differences resulted in amino acid substitutions. On the basis of these substitutions, we have correctly predicted the CQS/CQR status of a further 34 out of 36 isolates. This is a paradox as CQR arises much less frequently than would be predicted if single point mutations were sufficient. We conclude that

a mutated *pfmdr1* gene is one of at least two mutated genes required for CQR.

In initial studies we identified size polymorphisms of polymerase chain reaction (PCR)⁵ products in the 3' untranslated region of the *pfmdr1* gene that, surprisingly, were associated with CQR. Two different patterns of PCR products (designated the K1 and the 7G8 types after prototypic isolates) were associated with CQR (Fig. 1). Sensitive isolates had different polymorphic forms (Fig. 1). Sequence analysis demonstrated that these size polymorphisms are due to elongation of two (TA)_n repetitive regions. Secondary structure calculations predict that these repeats form a stable duplex, in accord with sequence evidence that the minor PCR bands are stable single stranded molecules.

To determine whether the 3' polymorphisms were linked to changes within the coding region, a number of *pfmdr1* genes were fully sequenced. The isolates chosen were two sensitive isolates (D10 and 3D7), two resistant isolates with the K1 type polymorphism (K1 and ITG2) and one with the 7G8 type polymorphism (7G8). Subsequently, two other full sequences were determined (see below). The sensitive isolates (D10 and 3D7) had identical *pfmdr1* genes. Amongst the resistant isolates, five key positions differed from the *pfmdr1* sequence of the sensitive isolates (Fig. 2). These were, first, Asn⁸⁶ to Tyr⁸⁶, the only change in both K1 type isolates (ITG2 and K1). 7G8 had four changes, Tyr¹⁸⁴ to Phe¹⁸⁴, Ser^{1,034} to Cys^{1,034}, Asn^{1,042} to Asp^{1,042} and Asp^{1,246} to Tyr^{1,246}. The only other changes were in a short polyasparagine 'hinge' segment² linking the halves of *pfmdr1* (data not shown). Tyr⁸⁶ is placed just before the third hydrophobic transmembrane segment, a position comparable to a change in the human *mdr1* gene which altered the substrate specificity of the gene product (P. glycoprotein)⁶. Two of the changes in the 7G8 *pfmdr1* gene occurred within the same transmembrane segment (Cys^{1,034} and Asp^{1,042}) and are both predicted to be on the hydrophilic side of an amphipathic helix. These changes are therefore found in domains of the molecule that may be involved in substrate specificity.

The association between these 'alleles' of the *pfmdr1* gene and CQR was tested by sequencing across the variant positions

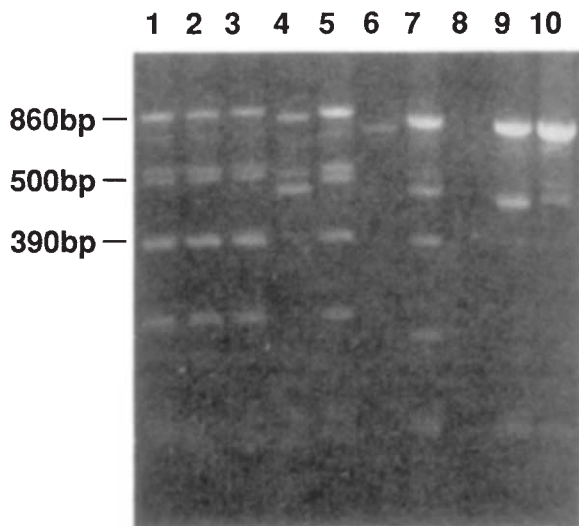


FIG. 1 Polymorphisms of PCR products from the 3' untranslated region correlate with CQR. The PCR's were performed using the oligonucleotides CGATCAGACAAATGTGGT and ATCAAAATATGTTACATGTG. The first is situated just within the coding region, the second 860 bp 3' to the first. The products of the PCR reaction were separated by agarose gel electrophoresis, stained with ethidium bromide and photographed under ultraviolet light. The sizes indicated are in base pairs. Lane 1, V1; lane 2, K1; lane 3, Csl-2; lane 4, 7G8; lane 5, IC1; lane 6, HB3; lane 7, D10; lane 8, blank; lane 9, 3D7; lane 10, Vietnam Smith.

Amino acid	86	184	1034	1042	1246
D10	Asn AAT.....	Tyr TAT.....	Ser AGT.....	Asn AAT.....	Asp GAT.....
3D7	Asn AAT.....	Tyr TAT.....	Ser AGT.....	Asn AAT.....	Asp GAT.....
K1	Tyr TAT.....	Tyr TAT.....	Ser AGT.....	Asn AAT.....	Asp GAT.....
ITG2	Tyr TAT.....	Tyr TAT.....	Ser AGT.....	Asn AAT.....	Asp GAT.....
7G8	Asn AAT.....	Phe TTT.....	Cys TGT.....	Asp GAT.....	Tyr TAT.....
Nig. 32	Tyr TAT.....	Phe TTT.....	Ser AGT.....	Asn AAT.....	Asp GAT.....
BT3	Asn AAT.....	Phe TTT.....	Ser AGT.....	Asp GAT.....	Asp GAT.....
nt	754	1049	3598	3622	4234

FIG. 2 Nucleotide changes and resultant amino acid changes identified through sequencing the entire *pfmdr1* gene from seven isolates of *P. falciparum*. The sequence of D10 is that presented in ref. 2. Overlapping fragments of the gene were amplified by PCR, separated from unincorporated oligonucleotides by agarose gel electrophoresis and purified from the low melting temperature agarose by hot phenol extraction. The PCR fragments were then directly sequenced¹⁵. The upper row of numbers refers to the amino acids (aa) and the lower to the nucleotide sequence (nt); numbering is from reference 2. Differences from the D10 and 3D7 sequences are represented in bold type. The only other differences between the isolates were variations in the number (+/-2) of asparagine residues in the polyasparagine tract of the 'hinge' region² and the silent point mutation in isolate Nigeria 32 (data not shown).

in CQR and CQS isolates after amplification by PCR. The 3' polymorphism was also examined (Table 1). From 36 isolates examined in a blind/blind study, the CQR/CQS status of 34 could be correctly predicted on the basis of the sequence alone (Table 1). In all, 48 isolates (including these 36) were investigated. All the resistant isolates established some years ago from South-East Asia had Tyr⁸⁶ and the K1 type 3' polymorphism (Table 1). Most of the CQR South American isolates, regardless of the date of isolation had the Phe¹⁸⁴/Cys^{1,034}/Asp^{1,042} and Tyr^{1,246} changes and the 7G8 type 3' polymorphism. The only exception was ITG2 from Brazil which had the K1 type allele. Phe¹⁸⁴ is probably not involved in CQR as it occurs in CQS isolates as well. We conclude that there are two resistant alleles, defined by Tyr⁸⁶ and by Cys^{1,034}/Asp^{1,042}/Tyr^{1,246} respectively. Vietnam-Smith appears to be a recombinant between these two forms as it has Tyr⁸⁶ but the 7G8 type 3' polymorphism (Table 1).

CQR probably arose independently in South-East Asia and South America and spread to the rest of the world. The two alleles identified above may represent two independent genetic events in South-East Asia and South America in the late 1950s that gave rise to the first CQR parasites^{7,8}. It is a paradox that CQR can be correctly predicted from the single base change in the K1 type allele. Single point mutations giving rise to CQR should arise as frequently as pyrimethamine resistance (caused by a point mutation in the dihydrofolate reductase gene)^{9,10} and this clearly has not happened. Therefore, we predict that both a *pfmdr1* allele competent for CQR and a mutation in a second unknown gene is required for CQR. This hypothesis can also account for the finding of Wellem's *et al.*¹¹, that *pfmdr1* did not segregate with CQR in a cross between CQR and CQS clones of *P. falciparum* if the *pfmdr1* genes of both clones were CQR-competent. A multigenic basis for CQR has also been demonstrated for rodent malaras made CQR *in vitro*^{12,13}.

CQR *P. falciparum* was detected in Africa 20 years after it arose in other parts of the world and analysis of the *pfmdr1* gene in recent CQR isolates from Nigeria revealed the 7G8 allele (Nigeria 14) as well as a novel allele (Nigeria 32 and Nigeria 63). This novel allele had the K1 type amino acid change

TABLE 1 Relationship between *pfmdr1* and CQR

Isolate*	Origin† & date of isolation	IC ₅₀ § (ng ml ⁻¹)	CQR/S	Prediction¶	3' poly-morphism	86#	184#	1,034#	1,042#	1,246#
D10‡	PNG (1979)	6††	S	ND		Asn	Tyr	Ser	Asn	Asp
3D7‡	Unknown	11††	S	ND		Asn	Tyr	Ser	Asn	Asp
D6‡	Africa (1979)	2.2‡‡	S	S		Asn	Tyr	Ser	Asn	Asp
Ghana	Africa	6.3††	S	S		Asn	Phe	Ser	Asn	Asp
HB3‡	S. America (1980)	10††	S	ND		Asn	Phe	Ser	Asp	Asp
K1	S.E. Asia	160††	R	ND	K1	Tyr	Tyr	Ser	Asn	Asp
IC1	S.E. Asia	141††	R	ND	K1	Tyr	Tyr	Ser	Asn	Asp
V1‡	S.E. Asia	185††	R	ND	K1	Tyr	Tyr	Ser	Asn	Asp
ITG2‡	S. America	71††	R	ND	K1	Tyr	Tyr	Ser	Asn	Asp
FCR3‡	Africa** (1976)	100††	R	R	K1	Tyr	Tyr	Ser	Asn	Asp
W2‡	S.E. Asia	60‡‡	R	R	K1	Tyr	Tyr	Ser	Asn	Asp
W2-Mef‡	S.E. Asia	36.2‡‡	R	R	K1	Tyr	Tyr	Ser	Asn	Asp
FCB	Africa	21.5‡‡	R	R	K1	Tyr	Tyr	Ser	Asn	Asp
Viet Smith	S.E. Asia	37.2‡‡	R	R	7G8	Tyr	Tyr	Ser	Asn	Asp
7G8‡	S. America	28.9‡‡	R	R	7G8	Asn	Phe	Cys	Asp	Tyr
Csl-2	S.E. Asia (1986)	78††	R	ND	K1	Tyr	Tyr	Ser	Asn	Asp
311‡	S. America (1984)	37.1‡‡	R	R	7G8	Asn	Phe	Cys	Asp	Tyr
1776	PNG (1987)	50††	R	R	K1	Tyr	Tyr	Ser	Asn	Asp
1787	PNG (1987)	10††	S	S		Asn	Tyr	Ser	Asn	Asp
1905	PNG (1987)	100††	R	R	7G8	Asn	Phe	Cys	Asp	Tyr
1933	PNG (1987)	89††	R	R	K1	Tyr	Tyr	Ser	Asn	Asp
1935	PNG (1987)	79††	R	R	K1	Tyr	Tyr	Ser	Asn	Asp
1775	PNG (1987)	56††	R	R	K1	Tyr	Tyr	Ser	Asn	Asp
1904	PNG (1987)	56††	R	R	K1	Tyr	Tyr	Ser	Asn	Asp
1916	PNG (1987)	8††	S	R	K1	Tyr	Tyr	Ser	Asn	Asp
1917	PNG (1987)	19††	S	S		Asn	Phe	Ser	Asn	Asp
1934	PNG (1987)	50††	R	R	K1	Tyr	Tyr	Ser	Asn	Asp
Nigeria 2	Africa (1989)	2.2‡‡	S	S		Asn	Phe	Ser	Asn	Asp
Nigeria 14	Africa (1989)	47‡‡	R	R	7G8	Asn	Phe	Cys	Asp	Tyr
Nigeria 16	Africa (1989)	1.8‡‡	S	S		Asn	Tyr	Ser	Asn	Asp
Nigeria 28	Africa (1989)	2.0‡‡	S	S		Asn	Tyr	Ser	Asn	Asp
Nigeria 32	Africa (1989)	19‡‡	R	R		Tyr	Phe	Ser	Asn	Asp
Nigeria 55	Africa (1989)	2.2‡‡	S	S		Asn	Phe	Ser	Asn	Asp
Nigeria 59	Africa (1989)	2.9‡‡	S	S		Asn	Tyr	Ser	Asn	Asp
Nigeria 60	Africa (1989)	29‡‡	R	S		Asn	Tyr	Ser	Asn	Asp
Nigeria 61	Africa (1989)	1.9‡‡	S	S		Asn	Phe	Ser	Asn	Asp
Nigeria 63	Africa (1989)	11‡‡	R	R		Tyr	Phe	Ser	Asn	Asp
306‡	S. America (1984)	26‡‡	R	R	7G8	Asn	Phe	Cys	Asp	Tyr
302‡	S. America (1984)	27‡‡	R	R	7G8	Asn	Phe	Cys	Asp	Tyr
IEC 55/84	S. America (1984)	16‡‡	R	R	7G8	Asn	Phe	Cys	Asp	Tyr
IEC 56/84	S. America (1984)	26‡‡	R	R	7G8	Asn	Phe	Cys	Asp	Tyr
IEC 54/84	S. America (1984)	23‡‡	R	R	7G8	Asn	Phe	Cys	Asp	Tyr
IEC 64/84	S. America (1984)	25‡‡	R	R	7G8	Asn	Phe	Cys	Asp	Tyr
313‡	S. America (1984)	20‡‡	R	R	7G8	Asn	Phe	Cys	Asp	Tyr
BT3	S.E. Asia (1989)	320§§	R	ND		Asn	Phe	Ser	Asp	Asp
Pf120	S.E. Asia (1989)	320§§	R	ND		Asn	Phe	Ser	Asn	Asp
Pf121	S.E. Asia (1989)	320§§	R	ND		Asn	Phe	Ser	Asp	Asp
Pf125	S.E. Asia (1989)	320§§	R	ND		Asn	Phe	Ser/Cys	Asp	Asp

* Parasites listed above isolate 311 are regarded in the text as being 'early', that is they were isolated in the early 1980s or before. The parasites below these were isolated over the last 2–3 years, except for the South American isolates.

† Specific information of these isolates can be found in refs 2, 9 and 10.

‡ Cloned parasite line

§ The 50% inhibitory concentration of chloroquine (IC₅₀) is that concentration of drug which inhibits growth of the parasites to a level 50% that of parasites grown in the absence of drug.

|| The clinical (CQR/S) status of the parasite is based on data presented in ref. 16 and on clinical observations of patients from whom parasites were isolated.

¶ The CQR/S status of the parasites was determined, DNA was prepared, coded and then given to one of us (S.F.) for sequencing and the prediction of the CQR/S status was then based solely on the predicted amino acids at positions 86, 1,034, 1,042 and 1,246.

The amino acid position of the variable residue, numbering refers to reference 2.

** The identity of this isolate is suspect as it was isolated from Africa before CQR was described on that continent. However the isolate used here has the FCR3 chromosome 1 deletion marker.

†† IC₅₀ analysis performed at the Walter and Eliza Hall Institute. Values here were consistently higher than those obtained at the Walter Reed Army Institute.

‡‡ IC₅₀ analysis performed at the Walter Reed Army Institute.

§§ These values are the mean inhibitory concentrations and were performed at Chulalongkorn University. They are not directly comparable to the other IC₅₀ values.

ND, Not determined.

(Tyr⁸⁶) but associated with Phe¹⁸⁴ and a different 3' polymorphism. The complete sequence of *pfmdr1* from Nigeria 32 showed only one other change, a silent mutation at nucleotide 2,656. This was the only silent mutation seen in any of the seven *pfmdr1* genes sequenced, apart from the hinge. One possibility is that

the Tyr⁸⁶ in the CQR African isolates is a new mutation, strengthening the claim that it has a role in CQR. On the basis of the two-gene hypothesis the presence of the 7G8 allele in a CQR isolate in Africa (Nigeria 14) would mean that a CQR competent form of the second gene is also present in the parasite

population. This would have allowed selection of the point mutation that gave rise to the *pfmdr1* gene of isolate Nigeria 32 in a sensitive parasite bearing this allele of the second gene. An example where only one of the two genes is CQR competent may be 1916 (PNG) which possesses a K1 type allele and yet is CQS. The low representation of CQS parasites that were predicted to be CQR from the *pfmdr1* DNA sequence is probably because 33 out of the 48 parasites were resistant and most of the CQS isolates came from regions where there was no CQR at the time of isolation.

Four multidrug-resistant isolates obtained from Thailand in the late 1980s were also examined. Some were mixed populations but with the exception of Pf120, all had the Asp^{1,042} and a subpopulation of Pf125 had Cys^{1,034}. The *pfmdr1* gene from BT3 was fully sequenced and, apart from a different length of the polyasparagine region differed from the CQS sequence only at Phe¹⁸⁴ and Asp^{1,042}. HB3 (sequenced in part) had the same changes as BT3 and yet is CQS. Perhaps the *pfmdr1* gene from HB3 is competent for CQR because of the Asp^{1,042} but, as hypothesized for 1916, lacks an appropriate allele of the second gene. In support of this idea, HB3 is less sensitive to mefloquine than other lines that we have tested (data not shown). Wellems *et al.*¹¹ performed their genetic cross between a subclone of isolate W2 (CQR, Tyr⁸⁶) and HB3. Their finding that CQR did not segregate with *pfmdr1* would be expected if the *pfmdr1* gene of HB3 was CQR competent. Pf120 and Nigeria 60 (both sequenced in part only) were the only isolates examined which had apparently wild type *pfmdr1* genes and yet were CQR. As neither of these were clones it is not clear that the DNA samples accurately represented the parasites assayed for CQR. Alternatively, they may represent new CQR alleles as they were not fully sequenced. Many of the more recent CQR isolates possessed new forms of the 3' polymorphism that were uninformative with regard to CQR.

The data presented here strongly links changes within the *pfmdr1* gene to CQR. As only one nucleotide differs between some CQS and CQR *pfmdr1* genes, we argue that CQR is a multigenic phenotype in order to explain the infrequent appearance of CQR and its slow geographical spread¹⁴. Two alleles of *pfmdr1* are found in CQR clones isolated a number of years ago. These may represent the initial events in South-East Asia and South America that gave rise to CQR. Amplification of *pfmdr1* in some isolates^{2,3} was presumably a later event selected for higher resistance as ITG2 and IC1 share the same allele but have amplicons of different size. Some later isolates have different mutations, although a recent mutation in some African isolates causes the same change as that in the early South-East Asian isolates. Knowledge of these mutations has allowed the correct prediction of the CQR/S status of a number of parasites. The data here has greatly increased the evidence that the *pfmdr1* gene is involved in CQR and may allow the basis for a rapid diagnostic test for CQR. □

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Selective killing of hepatitis B envelope antigen-specific B cells by class I-restricted, exogenous antigen-specific T lymphocytes

Vincenzo Barnaba, Alessandra Franco, Alfredo Alberti*, Rosalba Benvenuto & Francesco Balsano

Fondazione 'Andrea Cesalpino', I Clinica Medica, Policlinico 'Umberto I', Università 'La Sapienza', 00161 Roma, Italy

* II Clinica Medica, Istituto di Medicina Clinica, via Giustiniani 2, 35126 Padova, Italy

SPECIFIC B lymphocytes can act as very efficient antigen-presenting cells. They bind antigen with high affinity via their immunoglobulin receptors, process it through the class II major histocompatibility complex (MHC) pathway, and present its fragments to class II-restricted T lymphocytes¹. In general, exogenous antigens and noninfectious viral particles enter the class II pathway and are selectively associated with class II MHC molecules²⁻⁴. The presentation of an exogenous antigen in association with class I molecules has been reported for only a few antigens, including the hepatitis B envelope antigen (HBEnvAg)⁵⁻⁸. Here we demonstrate that antigen-specific B cells can efficiently deliver HBEnvAg to the class I pathway, presenting its fragments to class I-restricted cytotoxic T lymphocytes (CTLs) which kill the specific B cells. This could represent a mechanism of suppression of neutralizing anti-hepatitis B virus (HBV) antibody response, a phenomenon that accompanies the development of the chronic HBV-carrier state.

We have described previously human HLA A3-restricted CD8-positive and HLA DR2-restricted CD4-positive T cell clones, generated from lymphocytes infiltrating the liver during chronic hepatitis B⁸. Both class I- and class II-restricted T-cell clones proliferated in response to exogenous native HBEnvAg containing all three protein domains (pre-S1, pre-S2, and S) of the virus envelope, and recognized the same synthetic pre-S2 (120-134) peptide. To determine whether specific B cells were capable of presenting HBEnvAg to class I-restricted, as well as class II-restricted T lymphocytes, we isolated specific B cells from HLA-matched hepatitis B (HBV)-vaccine recipients and evaluated their ability to present low concentrations of antigen to both types of T cells. We tested these class I- and class II-restricted T-cell clones for specific lysis of MHC-restricted B cells bearing immunoglobulin receptors specific for HBEnvAg.

A DR2-restricted CD4⁺T clone and an A3-restricted CD8⁺T clone were compared for their ability to lyse specific and non-specific B cells that had been pulsed with different concentrations of HBEnvAg (Fig. 1a-d). Lysis of HBEnvAg-specific B cells by both class I- and class II-restricted pre-S2-specific T clones was obtained with very low concentrations (0.01 µg ml⁻¹) of antigen, whereas much higher concentrations (>50 µg ml⁻¹) were needed to induce a comparable level of killing of non-specific B cells. Lysis was antigen-specific, as pre-S2-specific T clones did not recognize another HBV antigen, the HB core antigen (HBcAg), even if it was presented by HBcAg-specific B cells (Fig. 1e, f). In addition, HBcAg-specific B cells presented HBEnvAg only at high concentration, similar to non-specific B cells (Fig. 1a, c). HBEnvAg-specific B cells did not present

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TABLE 1 MHC class I or class II-restriction of specific target B-cell lysis by cytotoxic T cells

Antibodies	T clone	Native HBEnvAg ($\mu\text{g ml}^{-1}$)	% Specific ^{51}Cr release
None	A10 (CD4)	0.1	42
Anti-class I	A10 (CD4)	0.1	40
Anti-class II	A10 (CD4)	0.1	9
None	C1 (CD8)	0.1	49
Anti-class I	C1 (CD8)	0.1	11
Anti-class II	C1 (CD8)	0.1	52

^{51}Cr -labelled specific target B cells were pulsed at 37°C (4 h) with $0.1 \mu\text{g ml}^{-1}$ native HBV-envelope, washed, and then incubated with MHC-restricted cloned T cells (4 h), used as effector cells at an E:T ratio 10:1, in the presence or absence of $10 \mu\text{g ml}^{-1}$ anti-class MHC antibody (W6/32, Cappel, Pennsylvania) or anti-class II MHC antibody (Q5/13 (ref. 24), provided by P. Giacomini, Cancer Institute, Rome). Per cent specific lysis is expressed as the mean of triplicate determinations.

HBcAg, which was not recognized by their surface antibodies (Fig. 1e, f).

Monoclonal antibodies against HLA-DR antigens and to HLA-A,B,C antigens blocked cytotoxicity against HBEnvAg-presenting B cells of pre-S2 specific class II- and class I-restricted T clones, respectively (Table 1). Addition of different concentrations of antibodies to hepatitis B surface antigen (anti-HBs) to nonspecific B cells, obtained from HBEnvAg-specific B cell supernatant, did not affect their susceptibility to lysis by pre-S2 specific T cells, ruling out the possibility that secreted antibodies participate in the high efficiency of antigen presentation by specific B cells (data not shown).

We next determined whether HBEnvAg, after binding to immunoglobulin receptors, was indeed processed by specific B cells to be presented to both class I- and class II-restricted T cells. We used chloroquine, which inhibits the endosomal processing pathway⁹, and paraformaldehyde fixation, which prevents all processing¹⁰, to study the presentation of low antigen concentration by specific B cells. Table 2 shows that chloroquine treatment of specific B cells, during pulsing with HBEnvAg, drastically reduced their susceptibility to lysis by class II-restricted specific cytotoxic T lymphocytes (CTLs), whereas no effect was seen for class I-restricted specific CTL reaction. As shown in Table 3, the paraformaldehyde-fixation of specific B cells before the antigen pulsing abolished the HBEnvAg presentation to both class II- and class I-restricted CTLs, although these B cells maintained the capacity of antigen binding through immunoglobulin. These data indicate that both class II- and class I-restricted T cells required some forms of antigen-processing by specific B lymphocytes. Control experiments show that specific B cells pulsed with HBEnvAg 2 h before fixation, or B cells pulsed with pre-S2 120–134 peptide after fixation, were efficiently killed by both types of CTLs, indicating that specific B cells remained lysable despite paraformaldehyde treatment (Table 3).

TABLE 2 Chloroquine effect on specific target B-cell sensitization by exogenous native HBEnvAg

Target cells	Chloroquine treatment	T clone	% Specific ^{51}Cr release
Specific EBV-line	—	A10 (CD4)	42
Specific EBV-line	+	A10 (CD4)	5
Specific EBV-line	—	C1 (CD8)	48
Specific EBV-line	+	C1 (CD8)	56

^{51}Cr -labelled specific target B cells were pulsed with $0.1 \mu\text{g ml}^{-1}$ HBEnvAg at 37°C (4 h) in the presence or absence of $100 \mu\text{M}$ of chloroquine (Sigma). The target cells were washed and then incubated with MHC-restricted CTLs at an E:T ratio 10:1. Per cent specific lysis is expressed as the mean of triplicate determinations.

The presentation of exogenous HBEnvAg with class I molecules has been previously described, but the mechanism is not clear⁷. HBEnvAg might contain peptides or linear fragments that bind directly to MHC molecules. In this case, if the specific B cell immunoglobulin receptor binds denatured HBEnvAg, then the antigen may be focused onto class I molecules without processing. Indeed, the antibodies derived from our specific B line reacted with pre-S2 peptide by enzyme-linked immunosorbent assay¹¹ and with denatured pre-S2 protein by western blotting. The results of fixation experiments argue against this possibility, however, and suggest that some form of processing is required. Processing could occur extracellularly¹² or involve the fusion of HBEnvAg with the membrane of antigen-presenting

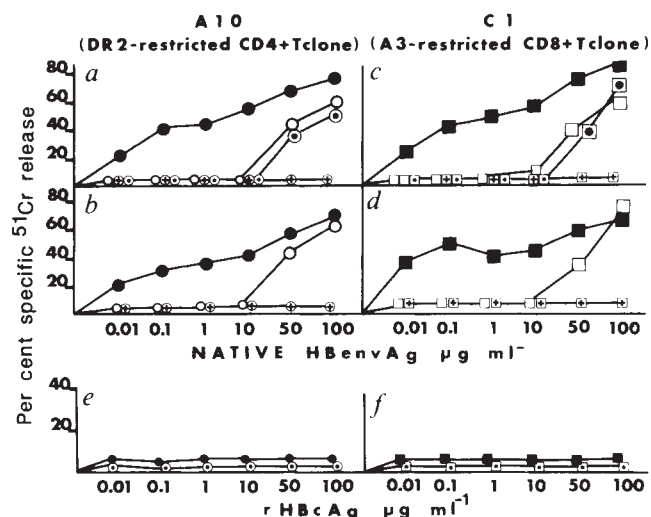


FIG. 1 Killing of specific B cells by class I-restricted CD8-positive or class II-restricted CD4-positive T cells induced by native HBEnvAg. *a* and *b*, Duplicate experiment to assess the cytotoxicity of a DR2-restricted pre-S2 specific CD4⁺ T clone (A10 clone; HLA-A2.3; B8.12; DR2.3) against B cells pre-pulsed with different concentrations of native HBEnvAg including: (●) DR2 positive allogeneic HBEnvAg-specific B cells (HLA-A1.1.1; B1.4; DR2); (○) DR2 positive autologous nonspecific B cells; (⊙) DR2 negative allogeneic HBEnvAg-specific B cells (HLA-A2.3; B8; DR3); (⊕) DR2 positive autologous HBcAg-specific B cells. *c* and *d*, Same experiments with an A3-restricted pre-S2 specific CD8⁺ T clone (C1 clone; HLA-A2.3; B8.12; DR2.3), and (■) A3 positive allogeneic HBEnvAg-specific B cells (HLA-A3; B12; DR4); (□) A3 positive autologous HBcAg-specific B cells; (⊞) A3 negative allogeneic HBEnvAg-specific B cells (HLA-A2; B8; DR3); (⊡) A3 positive autologous HBcAg-specific B cells. *e*, Cytotoxicity of pre-S2 specific CD4⁺ CTLs to (○) DR2 positive autologous HBcAg-specific B cells or (●) DR2 positive allogeneic HBEnvAg-specific B cells, pulsed with rHBcAg. *f*, Cytotoxicity of pre-S2 specific CD8⁺ CTLs to (□) A3 positive autologous HBcAg-specific B cells or (■) A3 positive allogeneic HBEnvAg-specific B cells, pulsed with rHBcAg.

METHODS. Plasma-derived HBEnvAg was prepared as described⁸. Pre-S2 specific T clones were generated from liver-infiltrating lymphocytes obtained from a chronic hepatitis B patient⁸. Briefly, intrahepatic lymphocytes were separated from hepatocytes by Ficoll-Hypaque density gradient centrifugation, washed, and plated (1×10^4 per well) in 96-well-bottomed plate (Falcon) with 1×10^5 autologous irradiated peripheral blood mononuclear cells (PBMC) as a source of antigen-presenting cells (APCs). HBEnvAg was added at $2-4 \mu\text{g ml}^{-1}$. After 7 days, cultures were cloned by limiting dilution at 0.3 cell per well with $1 \mu\text{g ml}^{-1}$ PHA-P (Wellcome), 10% IL-2 (Lymphocult, Biotech), and irradiated APCs. T clones showing specific HBEnvAg proliferation were maintained in culture by expansion in IL-2 containing medium, followed by periodic restimulations with PHA plus APCs every 2 weeks. The antigenic determinant recognized by T clones selected for these experiments mapped to the pre-S2 protein domain of HBV, based on analysis with recombinant antigens and synthetic peptides⁸. B-cell lines were obtained from the same donor of the T clones and from HB vaccine (HEVAC-B, Pasteur Institut) recipients by EBV-transformed peripheral blood B cells, and then distributed at cell concentrations of 500, 250, 125 and 62.5 cells per well in 96-well microplates. After 4 weeks, 7 out of 960 B cell lines from immunized subjects produced anti-HBs (IgG $> 1 \mu\text{g ml}^{-1}$) tested by ELISA, were then expanded and used as HBEnvAg-specific target B cells. None of 480 B cell lines derived from the chronic hepatitis B patient, donor of the specific T clones, produced anti-HBs. Some were expanded and used as autologous nonspecific target cells. By contrast, several of these last B cell lines produced antibodies to HBcAg (anti-HBc), as tested by ELISA, using the same rHBcAg used to pulse HBcAg-specific B cells for the antigen-specific cytotoxicity assays. One was expanded and used as autologous HBcAg-specific target cells. Antigen-specific cytotoxicity assay: ^{51}Cr -labelled EBV-transformed B cell lines, used as target cells, were pulsed at 37°C (4 h) with different concentrations of native HBEnvAg or recombinant (r)HBcAg and then washed. Cloned T cells, used as effector cells at an E:T ratio of 10:1, were incubated into 96-bottom microtitre wells in triplicate, to which 5×10^3 target cells were added and 4-h cytotoxicity assays performed. Per cent specific lysis is expressed as the mean of triplicate determinations.

TABLE 3 Paraformaldehyde fixation inhibits native HBenvAg processing, but not peptide presentation by specific target B cells

Target cells	Antigen pulsing		T clone	% Specific ⁵¹ Cr release
	Before fixation	After fixation		
Specific EBV-line	—	—	A10 (CD4)	6
Specific EBV-line	HBenvAg	—	A10 (CD4)	24
Specific EBV-line	—	HBenvAg	A10 (CD4)	3
Specific EBV-line	—	pre-S2 (120-134)	A10 (CD4)	32
Specific EBV-line	—	pre-S2 (153-171)	A10 (CD4)	5
Specific EBV-line	—	—	C1 (CD8)	7
Specific EBV-line	HBenvAg	—	C1 (CD8)	27
Specific EBV-line	—	HBenvAg	C1 (CD8)	5
Specific EBV-line	—	pre-S2 (120-134)	C1 (CD8)	40
Specific EBV-line	—	pre-S2 (153-171)	C1 (CD8)	6

⁵¹Cr-labelled specific target B cells were pulsed or not at 37 °C with 0.1 µg ml⁻¹ HBenvAg. After 2 h, the cells were washed and fixed with 0.5% paraformaldehyde (30 s) at room temperature. 0.2 r L-lysine (Sigma) was added to stop the fixation, the fixed cells were washed once, and then cultured with MHC-restricted CTLs at an E:T ratio 10:1. Native HBenvAg or 25 µg ml⁻¹ pre-S2 120-134 or 153-171 peptides, synthesized as described⁸, were added in cultures containing the fixed target B cells, which were not prepulsed with the native antigen. Fixed and unfixed specific B cells bound, respectively, 22,216 and 19,606 c.p.m. of ¹²⁵I-HBenvAg per 2 × 10⁶ cells, while fixed aspecific B cells bound 1,841 c.p.m. Per cent specific lysis is expressed as the mean of triplicate determinations.

cells and the delivery of the antigen into cytoplasmic class I-processing pathway^{6,12-14}. The fusion mechanism is of interest because HBenvAg is synthesized as a transmembrane protein inserted on the endoplasmic reticulum membrane, and contains host membrane lipids besides the S and pre-S proteins^{15,16}.

In exceptional cases, where an exogenous antigen is allowed access to the class I pathway, the specific focusing of the antigen by surface immunoglobulin can permit the presentation of processed fragments to class I-restricted CTLs, which in turn kill the B cells. Experiments with class II-restricted CTLs support lysis of specific B cells as a mechanism of suppression¹⁷⁻²¹. But there is no evidence as yet that class II-restricted T cells have any cytotoxic effect *in vivo*, although there is no doubt that class I-restricted T cells are indeed cytotoxic²². Suppression of antibody response by class II-restricted CTLs is speculative, but in the case of the HBenvAg, the selective killing of specific B cells by class I-restricted CTLs could have a role in suppressing the specific antibody response. Specific antibody suppression might provide a new explanation for the mechanism leading to chronic HBV infection, characterized by a defect in the antibody response to HBV-envelope proteins¹¹. Because these antibodies have a pivotal role in protective immunity against the virus²³, the lysis of the specific B cells by both class I- and class II-restricted specific CTLs, abundant in the liver during HBV infection, might participate in the establishment of a chronic HBV carrier state. □

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Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C

Mark S. Perin*, Victor A. Fried†, Gregory A. Mignery*‡, Reinhard Jahn§ & Thomas C. Südhof*‡||

*Howard Hughes Medical Institute and ‡Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, Texas 75235, USA

†St Jude Children's Research Hospital, Department of Biochemistry, Memphis, Tennessee 38101, USA

§MPI für Psychiatrie, Abteilung Neurochemie, 8033 Planegg-Martinsried, FRG

NEUROTRANSMITTERS are released at synapses by the Ca²⁺-regulated exocytosis of synaptic vesicles, which are specialized secretory organelles that store high concentrations of neurotransmitters^{1,2}. The rapid Ca²⁺-triggered fusion of synaptic vesicles is presumably mediated by specific proteins that must interact with Ca²⁺ and the phospholipid bilayer. We now report that the cytoplasmic domain of p65, a synaptic vesicle-specific protein³ that binds calmodulin⁴ contains an internally repeated sequence that is homologous to the regulatory C2-region of protein kinase C (PKC)⁵. The cytoplasmic domain of recombinant p65 binds acidic phospholipids with a specificity indicating an interaction of p65 with the hydrophobic core as well as the headgroups of the phospholipids. The binding specificity resembles PKC, except that p65 also binds calmodulin, placing the C2-regions in a context of potential Ca²⁺-regulation that is different from PKC. This is a novel homology between a cellular protein and the regulatory domain of protein kinase C. The structure and properties of p65 suggest that it may have a role in mediating membrane interactions during synaptic vesicle exocytosis.

Rat synaptic vesicles were purified by centrifugation and controlled-pore glass chromatography, and p65 was isolated by preparative SDS-PAGE⁶. Purified p65 was digested with trypsin and individual fragments were isolated by HPLC and subjected to amino acid sequencing⁶. The amino acid sequences were then used to design short oligonucleotides that were employed in the polymerase chain reaction^{7,8} with rat genomic DNA to generate a larger hybridization probe, which in turn was used to isolate overlapping complementary DNA clones encoding p65 (Fig. 1).

The messenger RNA for p65 has unusually long 3' and 5' untranslated regions and contains a single long open reading frame that predicts translation of a 421 residue protein (Fig. 1). Hydrophobicity plots of the protein sequence of p65 reveal a single segment of sufficient length and hydrophobicity to constitute a transmembrane region (broken line in Fig. 1), possessing boundaries of an unusual nature. On the N-terminal border there is a cluster of three prolines that may be extra- or intramembranous. At the C-terminal side, there is a cysteine-rich sequence

|| To whom correspondence should be addressed.

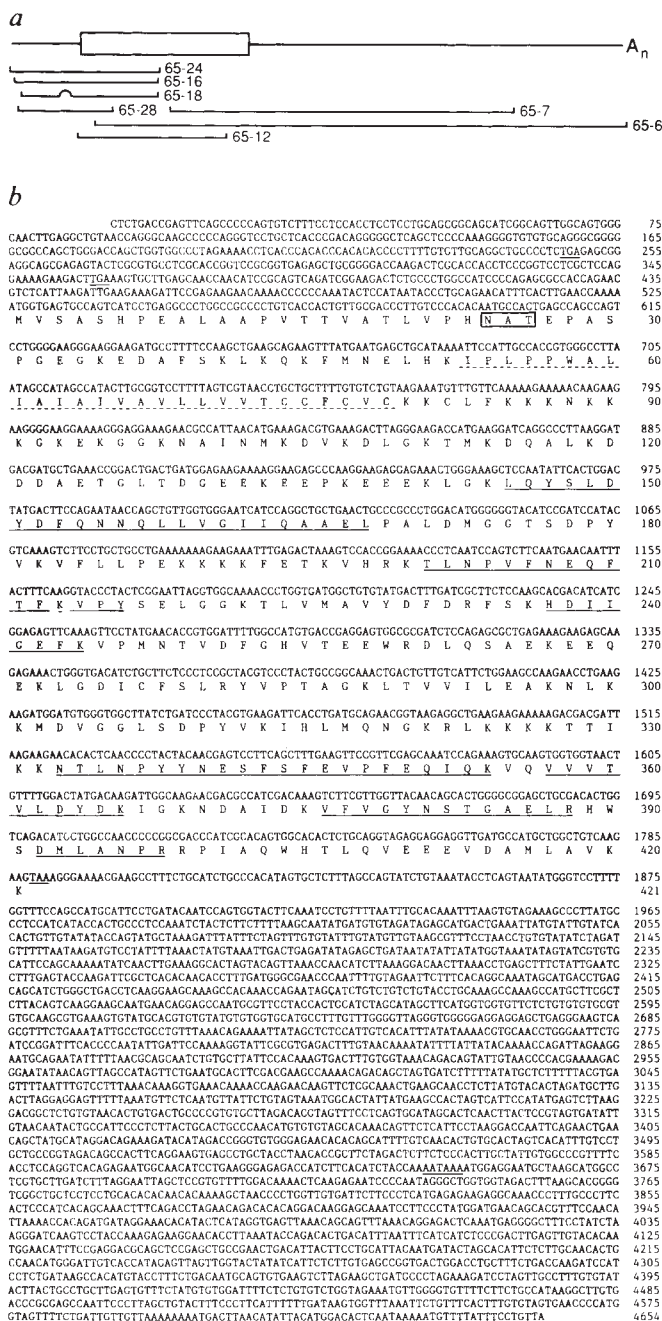


FIG. 1 Structure of p65. **a**, Diagram of p65 mRNA and positions of cDNA clones. The open box represents the location of the coding region. **b**, Nucleotide and translated amino acid sequences of p65. Two in-frame stop codons in the 5' untranslated region are underlined, as well as the translation termination codon and two polyadenylation consensus sequences in the 3' untranslated region. Amino acid sequences obtained from p65 trypsin fragments are indicated by solid underlines, and the putative transmembrane region by a broken line. The single consensus sequence of *N*-linked glycosylation in the intravesicular portion of p65 is boxed. The nucleotide and protein sequences are numbered on the right.

METHODS. p65 was purified from isolated synaptic vesicles by preparative SDS-PAGE⁶, trypsinized and subjected to HPLC. Sequences obtained from isolated proteolytic fragments were used to design oligonucleotide primers for PCR^{7,8}, the results of which were then used to synthesize a long specific probe that was used to screen a rat brain cDNA library. Additional cDNAs were obtained by rescreeing the library with a more 5' oligonucleotide. The nucleotide sequences of most clones were obtained on both strands except for 65-7, of which only parts were sequenced. The following sequence differences were observed between cDNA clones: nucleotides 345 to 445 were absent in 65-18; base 820 is G in 65-6, changing Asn(99) to Asp; base 871 is G in 65-12, changing Gln to Glu; bases 874 to 883 are missing in 65-16 and 65-24 but present in 65-6, 65-12 and 65-18, causing a three amino acid deletion in two of the five cDNA clones sequenced in this region; base 1,030 is G in 65-6, changing amino acid Pro(168) to Ala; and base 1,221 is G in 65-12, changing Asp (232) to Glu. The reasons for these differences are at present not known. The Lys residue at position 213 was an ambiguous residue during sequencing and was not cleaved by trypsin, suggesting that it may be post-translationally modified.

isoforms of PKC, reveals that the repeats are only slightly more homologous to each other (41% identity) than to the corresponding regions of PKC (identity between 32 and 40% depending on the isoform, Fig. 2b). Those residues that are identical between the internal p65 repeats tend to be more conserved between p65 and PKC. Non-identical residues often represent conservative changes, particularly if they are hydrophobic, indicating a similar conformation of these domains (Fig. 2b).

Several different isoforms of PKC have been described, all of which contain a catalytic C-terminal domain that is homologous to that of other protein kinases, and an N-terminal regulatory domain that is unique to PKC (reviewed in ref. 5). The activity of most forms of PKC is dependent on phosphatidyl-

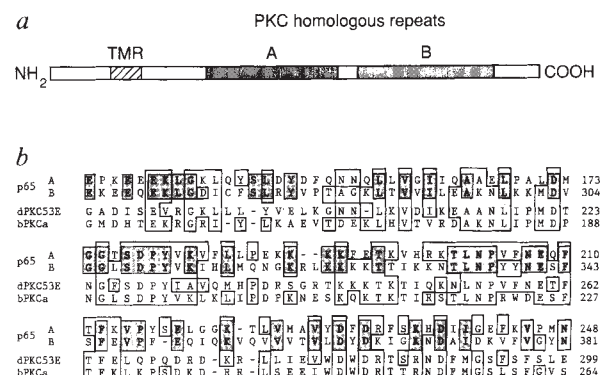


FIG. 2 Repeat structure of p65. **a**, Domain model of p65. The short sequence that is N-terminal to the transmembrane region (TMR) is thought to be intravesicular, and the sequences following the TMR cytoplasmic. **b**, Alignment of the two internal repeats in the cytoplasmic portion of p65 (A and B, top two lines) with the C₂-domain sequences of two isoforms of PKC (third line, photoreceptor specific form of PKC from *Drosophila melanogaster*¹⁹, and fourth line, bovine PKC-α¹⁰). Residues shared between the two internal repeats of p65 are highlighted by shaded boxes, while residues that are identical between any of the PKC sequences and the p65 sequences are boxed. Residue numbers are given on the right.

followed by a cluster of positive charges (8 lysine residues in 11 conserved amino acids).

A monoclonal antibody directed against p65³ reacts with sequences on the cytoplasmic side of synaptic vesicles (M.S.P. *et al.*, manuscript in preparation). This antibody also reacts with a recombinant p65 protein expressed in *Escherichia coli* that lacks the first 78 amino acids of the protein including the transmembrane region (see below), indicating that the large sequence following the transmembrane region is cytoplasmic.

The cytoplasmic sequence of p65 contains two copies of a 116 amino acid repeat that share 41% identity (Fig. 2a). The two repeats are separated from the transmembrane region by a highly charged sequence that contains four copies of the triplet X-Lys-Asp, where X is a hydrophobic amino acid. The internal repeats of p65 were found to be homologous to the regulatory domain of PKC. Alignment of the two internal p65 repeats with each other and with the regulatory domain of several different

TABLE 1 Inhibition by lipids of haemagglutination by recombinant p65

Compound	Minimal inhibitory concentration	
Phosphatidylserine	0.4 $\mu\text{M} \pm 0$	4
Phosphatidylinositol	0.6 $\mu\text{M} \pm 0.2$	4
Sulphatides	0.4 $\mu\text{M} \pm 0$	4
Phosphatidylglycerol	0.4 $\mu\text{M} \pm 0$	4
Trisialoganglioside (GT1b)	0.9 $\mu\text{M} \pm 0.5$	7
Disialoganglioside (GD1)	3.9 $\mu\text{M} \pm 1.4$	4
Monosialoganglioside (GM3)	7.8 $\mu\text{M} \pm 2.7$	4
Monosialoganglioside (GM2)	34 $\mu\text{M} \pm 16.0$	4
Monosialoganglioside (GM1)	19 $\mu\text{M} \pm 8.0$	5
Lysophosphatidylserine	200 $\mu\text{M} \pm 0$	4
Lysophosphatidylinositol	>200 μM	4
Lysophosphatidylglycerol	>200 μM	4
Lysosulphatides	>200 μM	4
Phosphatidylethanolamine/ phosphatidylcholine	>200 μM	4
Diacylglycerol	>200 μM	4
Cerebrosides	>200 μM	4
Oleic acid	>100 μM	4

Glutaraldehyde-treated rabbit erythrocytes were trypsinized and washed¹⁷. Haemagglutination assays were performed at 23 °C in microtitre plates in 100 μl volumes, containing 0.15 M NaCl, 10 mM Tris-HCl (pH 7.4), 2.5 g l⁻¹ BSA, 1% packed erythrocytes, 0.025 g l⁻¹ SDS, and 2.4 mg l⁻¹ of the recombinant cytoplasmic domains of p65 (residues 78–421; Fig. 1) that was solubilized in 1% SDS and extensively dialysed against 100 mM NaCl, 10 mM HEPES-NaOH (pH 7.4). The concentration of recombinant p65 constitutes 2–4 times the minimal concentration necessary to achieve haemagglutination (~30–60 nM). Compounds were titrated for their inhibitory activity by serial dilutions, the minimal inhibitory concentration being the minimal concentration of the inhibitory compound that completely blocked haemagglutination. For compounds that did not inhibit the reaction, the largest concentration tested is shown. The number of experiments is indicated (N). Phospholipids (Avanti Lipids) were added as small unilamellar vesicles; lysophospholipids (Avanti Lipids, except for lysophosphatidylglycerol and lyso-sulphatides, Sigma) and sphingolipids and lysosphingolipids (gangliosides were from Boehringer; cerebrosides from Avanti Lipids; sulphatides and lysosulphatides were from Sigma) were added as micelles. Oleic acid (Sigma) and diacylglycerol (Avanti Lipids) were added as suspensions; all other compounds (Sigma) were added as solutions. In all assays, negative controls contained protein from nonexpressing bacteria or from bacteria expressing an irrelevant protein (rab3^{18,20}). Positive controls contained soybean haemagglutinin (E.Y. Laboratories, California).

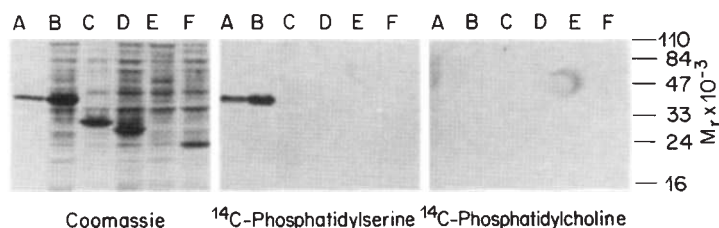
serine, diacylglycerol or phorbol esters, and calcium (reviewed in ref. 9). The regulatory domain of PKC contains two sub-domains, an N-terminal cysteine-rich C₁-domain that is present in all species of PKC, and a C-terminal C₂-domain that is absent from some forms of PKC^{10–13}.

Phorbol esters and diacylglycerol bind to the C₁-regulatory region of PKC¹⁴, but the phosphatidylserine and Ca²⁺-binding sites of PKC are not known. However, the isoforms of PKC that lack the C₂-regulatory region have a Ca²⁺-independent kinase activity^{12,13}, suggesting that the C₂-region contains a Ca²⁺-binding site. The homology of the internal repeats of p65 to PKC coincides with the end of the C₁-regulatory region and covers almost all of the C₂-regulatory domain. No homology was observed between p65 and those species of PKC lacking a C₂-regulatory domain, suggesting that the C₂-regulatory domain represents a separate functional module shared by some forms of PKC and p65.

The sequence homology of p65 to the regulatory C₂-domain of PKC indicates that p65 may bind phospholipid or Ca²⁺. To investigate these possible functional activities, we expressed the cytoplasmic domain of p65 in *E. coli*. Bacteria transformed with a p65 expression construct containing residues 78–421 (Fig. 1) synthesized a protein of relative molecular mass (M_r) 42,000, that reacted specifically with a monoclonal antibody directed against the cytoplasmic domain of p65³ (data not shown). The recombinant protein was tested by ligand blotting for phosphatidylserine and Ca²⁺ binding. Phosphatidylserine bound strongly, whereas phosphatidylcholine did not (Fig. 3). No Ca²⁺ binding could be detected (data not shown).

The presence of a duplication of the C₂-regulatory domain of PKC in p65 suggests that p65 may be capable of bivalent interactions. A haemagglutination activity has been described that copurifies with synaptic vesicles and is inhibited by gangliosides¹⁵. The recombinant cytoplasmic portion of p65 was tested for its ability to agglutinate trypsinized rabbit erythrocytes. A strong activity was found that was specific for p65 and active at concentrations less than ~10 nM of the recombinant protein. The agglutination of rabbit erythrocytes did not require calcium or diacylglycerol, but could be specifically inhibited by negatively charged phospholipids and sphingolipids, including gangliosides, at concentrations below 1 μM (Table 1). Phosphatidylserine specifically inhibited haemagglutination of rabbit erythrocytes since no effect was observed on the haemagglutination produced by soybean agglutinin. Negatively charged phos-

FIG. 3 Binding of ¹⁴C-phosphatidylserine but not ¹⁴C-phosphatidylcholine to the cytoplasmic portion of recombinant p65. Total proteins from bacteria harbouring different vectors were separated by SDS-PAGE and either stained with Coomassie blue (left panel) or blotted and reacted with ¹⁴C-phosphatidylserine (middle panel) or ¹⁴C-phosphatidylcholine (right panel). Lanes were loaded with protein from bacteria containing the following constructs: lanes A and B, a construct encoding residues 78–421 of p65 (12.5 and 50 μg protein, respectively); lanes C and D, constructs encoding two different segments of the rat InsP₃-receptor (P.A. Johnston *et al.*, manuscript in preparation); lane E, the expression vector only; and F, a construct encoding rab3^{18,20} (50 μg protein in lanes C to F). METHODS. An expression plasmid encoding residues 78–421 of p65 under the control of the T7 promoter was constructed by PCR using oligonucleotide primers complementary to the ends of the cDNA sequence encoding this peptide sequence. The 5' primer contained an initiator codon in the context of an *Nco*I site, and the 3' primer incorporated a *Bgl*II site after the termination codon. The resulting PCR fragment⁷ was cut with *Nco*I and *Bgl*II and cloned into the *Nco*I and *Bam*HI sites of plasmid pET8c. Recombinant plasmids were transformed into *E. coli* BL21 (DE3) cells, and protein expression was induced with 0.4 mM IPTG for 2 h. Constructs encoding



different parts of the rat InsP₃-receptor and rab3 were obtained and expressed in a similar manner. Nitrocellulose blots of the bacterial proteins separated by SDS-PAGE were preblocked for 30 min in 30 g l⁻¹ BSA, 0.2 g l⁻¹ phosphatidylserine/phosphatidylethanolamine (1:1, w/w), 130 mM NaCl, and 20 mM HEPES-NaOH (pH 7.4) for 30 min at room temperature. After washes for 10 min in 130 mM NaCl, 20 mM HEPES-NaOH (pH 7.4), the blots were reacted with 10 μM ¹⁴C-phosphatidylserine/phosphatidylethanolamine or with ¹⁴C-phosphatidylcholine/phosphatidylethanolamine (2 $\times$ 10⁵ c.p.m. ml⁻¹) for 30 min at room temperature, washed 5 min in 130 mM NaCl, 20 mM HEPES-NaOH (pH 7.4), dried and exposed to film (30 h exposure). All lipids were added as unilamellar liposomes.

pholipids and sphingolipids inhibited haemagglutination, but their lyso-derivatives containing only one acyl chain did not (Table 1). Neutral zwitterionic phospholipids were equally ineffective, as were various sugars, sphingosine, phorbol esters, diacylglycerol, trifluoperazine and calmodulin at concentrations of up to 10 μ M (Table 1).

Our results show that the cytoplasmic domain of p65 binds acidic phospho- and sphingolipids with high affinity (Table 1). Recombinant p65 has little specificity for the lipid headgroup besides negative charges, but only binds acidic phospholipids containing two acyl chains and not one acyl chain. This implies that the recombinant protein makes contact with the hydrophobic portion of the phospholipids and perhaps inserts partially into the bilayer. The binding specificity of p65 for

negatively charged diacyl lipids resembles that of the membrane binding of PKC, although the localization and number of phospholipid binding sites in PKC is not known¹⁶. The relation between calmodulin and possibly Ca^{2+} -binding and the phospholipid-interaction of p65 described here is currently unclear.

The structure of p65 demonstrates that at least one of the regulatory domains of PKC is shared with other intracellular proteins, a situation that may have evolved by exon shuffling. The duplication of such a domain makes p65 capable of bivalent interactions. The possible interaction of p65 with the hydrophobic interior of the phospholipid bilayer indicates that it may insert into and structurally modulate the bilayer. Together these results suggest a role for this protein in synaptic vesicle exocytosis. □

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Cytoplasmic dynein is localized to kinetochores during mitosis

C. M. Pfarr*, M. Coue*, P. M. Grissom*, T. S. Hays†, M. E. Porter‡ & J. R. McIntosh*

* Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309, USA

† Department of Genetics and Cell Biology, University of Minnesota, St. Paul, Minnesota 55108, USA

‡ Department of Cell Biology and Neuroanatomy, University of Minnesota, Minneapolis, Minnesota 55455, USA

RECENT evidence suggests that the force for poleward movement of chromosomes during mitosis is generated at or close to the kinetochores^{1,2}. Chromosome movement depends on motion relative to microtubules, but the identities of the motors remain uncertain^{1,3-7}. One candidate for a mitotic motor is dynein, a large multimeric enzyme which can move along microtubules toward their slow growing end⁸⁻¹¹. Dyneins were originally found in axonemes of cilia and flagella where they power microtubule sliding. Recently, cytoplasmic dyneins have also been found¹², and specific antibodies have been raised against them. The cellular localization of dynein has previously been studied with several antibodies raised against flagellar dynein, but the relevance of these data to the distribution of cytoplasmic dynein is not known¹³⁻¹⁷. Antibodies raised against cytoplasmic dyneins have shown localization of dynein antigens to the mitotic spindles in *Caenorhabditis elegans* embryos (Lye *et al.*, personal communication) and punctate cytoplasmic structures in *Dictyostelium amoebae*¹⁸. Using antibodies that recognize subunits of cytoplasmic dyneins, we show here that during mitosis, cytoplasmic dynein antigens concentrate near the kinetochores, centrosomes and spindle fibres of HeLa and PtK₁ cells, whereas at interphase they are distributed throughout the cytoplasm. This is consistent with the hypothesis that cytoplasmic dynein is a mitotic motor.

Cytoplasmic dynein was purified from HeLa cells grown in suspension culture (Fig. 1)¹⁹. It includes a heavy-chain subunit of $M_r \sim 420,000$ (420K) which can be cleaved into two fragments by irradiation with near ultraviolet light in the presence of

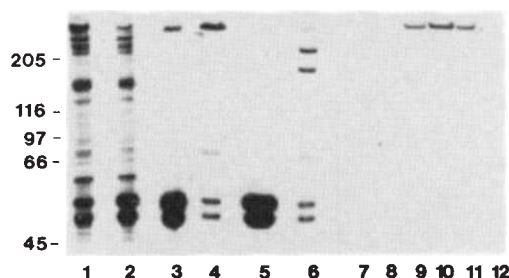


FIG. 1 Purification of cytoplasmic dynein from HeLa Cells. Lane 1, Biogel A5.M chromatographic fraction enriched in dynein heavy chain polypeptides. Supernatant (lane 2) and pellet (lane 3) after exogenous taxol-stabilized microtubules were incubated with the column fraction in Lane 1 and centrifuged. Supernatant (lane 4) and pellet (lane 5) after the pellet in Lane 3 was extracted with MgATP. Relative loading of sample in Lane 4 is five times that of lanes 3 and 5. Lane 6, cleavage of heavy chain polypeptides by ultraviolet light result in fragments of 190 and 230K. Lanes 7-12, fractions from a sucrose gradient showing cosedimentation of the 79 and 420K polypeptides. Migration of M_r markers (in thousands) is shown on the left.

METHODS. HeLa cells grown in suspension and collected by centrifugation were resuspended in buffer of 10 mM Tris, pH 7.5, 10 mM KCl, 2 mM MgSO_4 , and 1 mM DTT plus protease inhibitors: 5 $\mu\text{g ml}^{-1}$ each of leupeptin, aprotinin, and pepstatin A, 0.1 mg ml^{-1} soybean trypsin inhibitor, and 1 mM phenylmethylsulphonyl fluoride (PMSF). Cells were homogenized and centrifuged at $10^5 g$ for 1 h at 4 °C. $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to 55% saturation, and the precipitated protein was collected by centrifugation. The pellet was resuspended in column buffer (50 mM HEPES, 50 mM PIPES, pH 7.2, 2 mM MgSO_4 , 1 mM EDTA, and 0.5 mM DTT, plus protease inhibitors) and loaded onto a 2.5 $\times$ 50 cm Biogel A5.M column. The fractions enriched in dynein heavy-chain polypeptides were brought to 10 μM in taxol, and taxol-stabilized, phosphocellulose-purified, bovine brain microtubules were added to a final concentration of 2 mg ml^{-1} . After incubation at 20 °C for 1 h the sample was centrifuged through a 10% sucrose cushion. The pellet was resuspended in column buffer containing 10 mM ATP, 10 mM MgSO_4 , and 10 μM taxol, incubated on ice for 30 min and the microtubules re-pelleted. The ATP extracts were further purified by centrifugation on 5-20% sucrose density gradients made in column buffer. For UV cleavage, ATP extracts were supplemented with 10 μM Na_2VO_4 , and irradiated with UV light for 30 min at 4 °C. Samples were analysed on a 7% SDS-polyacrylamide gel and stained with Coomassie blue.

vanadate ions and ATP (Fig. 1, lane 6)²⁰. A smaller polypeptide of $M_r \sim 79,000$ (79K) co-pellets with the 420K subunit, and is similarly extracted from microtubules with MgATP (Fig. 1, lane 4). This 79K polypeptide co-purifies with the 420K subunit by sucrose density gradient centrifugation (Fig. 1, lanes 7–12) with a constant stoichiometry of 1:1. The 79K polypeptide is therefore a probable subunit of HeLa cytoplasmic dynein.

Dynein purified on a sucrose density gradient (Fig. 1, lane 10) and adsorbed to a glass coverslip will support microtubule gliding at a rate of $1\text{--}3\ \mu\text{m s}^{-1}$. Motility is dependent on MgATP and is directed toward the minus (slow-growing) end of the microtubule¹⁹. Movement is inhibited by $5\ \mu\text{M}$ vanadate and is blocked if the 420K subunit is subjected to cleavage by ultraviolet light (UV).

A rabbit polyclonal antiserum was raised against the heavy chain of cytoplasmic dynein from *Drosophila* embryos by injection of bands excised from SDS-polyacrylamide gels (Hays *et al.*, personal communication). This antiserum cross-reacted with

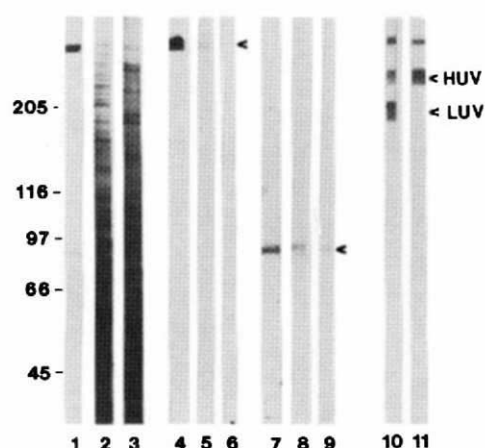


FIG. 2 Immunoblot analysis of antibodies against HeLa cytoplasmic dynein. Lanes 1–3 are Coomassie-stained gel lanes of an ATP extract enriched in HeLa dynein, whole HeLa cell protein, and whole PtK₁ cell protein, respectively. Lanes 4–6 are immunoblots of samples equivalent to lanes 1–3 probed with affinity-purified antibodies against *Drosophila* embryo cytoplasmic dynein heavy chain (antibodies R-1). Lanes 7–9 are immunoblots of samples equivalent to lanes 1–3 probed with affinity-purified antibodies against HeLa cytoplasmic dynein 79K intermediate chain (antibodies C1). Lanes 10 and 11 are immunoblots of a UV-cleaved dynein preparation (equivalent to Fig. 1, lane 6). Lane 10 is probed with R-1 antibodies which recognize both the heavier (HUV) and lighter (LUV) cleavage fragments and the intact heavy chain. Lane 11 is probed with antibodies affinity purified against only the HUV fragment. Migration of M_r markers is shown on the left (in thousands). METHODS. Antibodies were raised in a New Zealand white rabbit by injection of *Drosophila* embryo dynein heavy chains cut from 7.5% SDS-polyacrylamide gels. Gel bands were emulsified with complete Freund's adjuvant for the initial immunization, and booster injections were given at 2-week intervals with antigen mixed with incomplete adjuvant. Chicken antibodies against the 79K dynein subunit were raised using a similar protocol. Antibodies were isolated from egg yolks by polyethylene glycol precipitation²². Rabbit antisera and chicken egg yolk IgYs were affinity purified against antigens blotted onto nitrocellulose sheets²³. For immunoblot testing, the purified antibodies were diluted 1:1 in 10 mM Tris, pH 7.0, 10 mM NaCl, 0.05% Tween-20 (TST), plus 10% fetal calf serum, then incubated with the test strip for 10–12 h at 4 °C with agitation. Strips were washed and incubated for 1 h at 37 °C with a secondary antibody conjugated to horseradish peroxidase followed by development with 4-Cl-naphthol and H₂O₂ in TST. Preimmune rabbit sera and chicken egg IgYs were affinity purified and tested in an identical manner. The purified preimmune antibodies produced no detectable signal on immunoblots of lanes 1–3 (data not shown). For whole cell protein samples, HeLa cells from suspension culture were collected by centrifugation, washed twice in PBS and put directly into boiling sample buffer (lane 2). PtK₁ whole cell protein was similarly prepared except that the cells were grown as monolayers and collected by scraping with a rubber policeman (lane 3). The samples were run on a 7% SDS-polyacrylamide gel and stained with Coomassie blue, or blotted to nitrocellulose.

the heavy-chain subunit of HeLa cytoplasmic dynein. Affinity purification against HeLa heavy-chain polypeptides generated

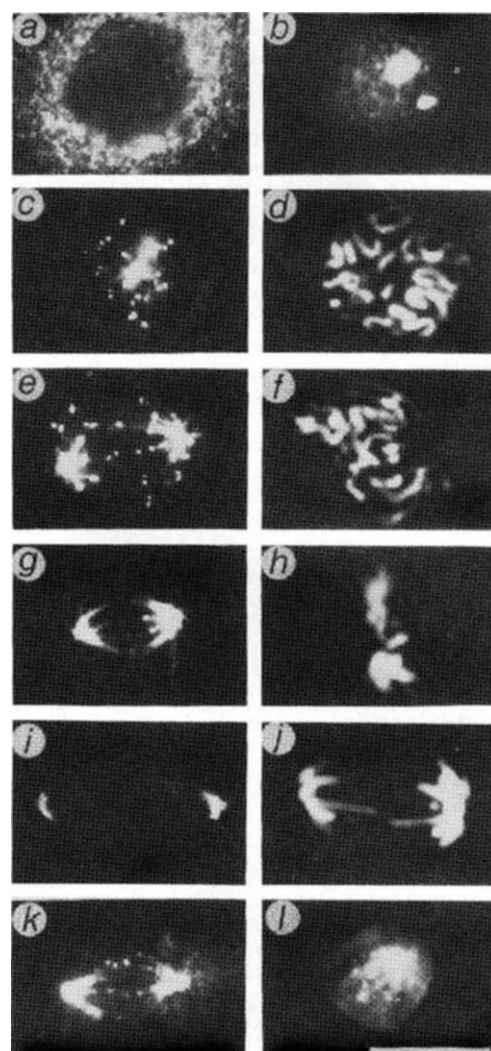


FIG. 3 a–j, Immunofluorescence of PtK₁ cells labelled with R-1 antibodies (left column) and 4',6-diamidino-2-phenylindole (DAPI) (right column). a, Interphase cell showing punctate cytoplasmic staining, but no intranuclear dynein signal; c, late prophase cell with bright dynein staining at kinetochores and spindle poles (seen as two diffusely stained regions toward the centre); e, prometaphase cell showing increased dynein staining of spindle fibres; g, metaphase cell with dynein labelling concentrated between the chromosomes and the poles; i, late anaphase cell with kinetochores clustered around their respective poles; k, metaphase PtK₁ cell labelled with C-1 antibodies. Note similarity to metaphase cell in g; l, prometaphase HeLa cell labelled with R-1 antibodies. Because the cell rounds up during mitosis only one spindle pole is visible. All cells were lysed with detergent and fixed in aldehyde. Similar staining was observed in cells fixed in methanol but spindle detail was less clear (data not shown). Scale bar 20 μm .

METHODS. HeLa and PtK₁ cells were grown as monolayers on glass coverslips. Cells were lysed for 2 min in a buffer (PHEM) containing 60 mM PIPES, 25 mM HEPES, pH 7.1, 2 mM MgSO₄, 10 mM EGTA plus 0.2% Triton X-100. The cells were fixed for 20 min in PHEM buffer containing 0.1% glutaraldehyde and 2% paraformaldehyde followed by quenching in PHEM containing 10 mg ml⁻¹ NaBH₄ for 5 min and several rinses in TST. For MeOH fixation, cells were immersed in MeOH at –20 °C for 5 min, followed by acetone at –20 °C for 7 min and several rinses in TST. The fixed cells were incubated with the affinity-purified antibodies at dilutions from 1:1 to 1:10 for 10–12 h at 5 °C in TST containing 10% fetal calf serum. Secondary antibodies (Texas Red conjugated goat anti-rabbit and rabbit anti-chicken; Jackson Immunochemicals) were incubated for 1 h at 37 °C in TST plus 10% fetal calf serum. Cells were rinsed in TST, stained with 0.05% DAPI and mounted for viewing. Micrographs were taken on a Zeiss Universal microscope with a 100 $\times$ phase contrast objective and recorded on Kodak Technical Pan 2415 film developed in HC-110.

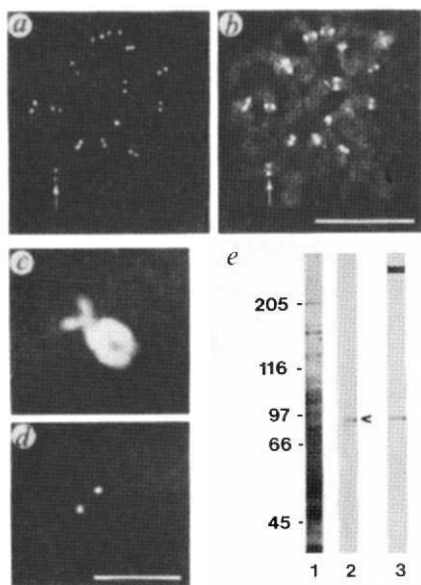


FIG. 4 Localization of cytoplasmic dynein to kinetochores. *a*, Prophase PtK₁ cell stained with CREST anti-kinetochore antibodies; *b*, same cell stained with the R-1 antibodies plus DAPI. The anti-dynein and CREST signals are seen to co-localize at pairs of kinetochores (arrows); *c*, isolated HeLa mitotic chromosome stained with DAPI; *d*, same chromosome labelled with C-1 antibodies; *e*, lanes 1 and 3 are Coomassie-stained gel lanes containing HeLa chromosomal protein and an ATP extract enriched in dynein, respectively. Lane 2 is an immunoblot corresponding to lane 1, showing that C-1 antibodies react with a 79K chromosomal protein. Migration of *M_r* markers is indicated. *a*, *b*, scale bar 10 μ m, *c*, *d*, 2 μ m.

METHODS. Immunofluorescence staining is as described in Fig. 3. CREST and R-1 antibodies were visualized with Texas Red and fluorescein-conjugated secondary antibodies respectively. For isolation of chromosomes, HeLa cells were grown as monolayers and blocked in mitosis by treatment with 10 μ g ml⁻¹ vinblastine sulphate for 12–15 h. After a mitotic shake-off, the cells were collected by centrifugation, lysed, and fractionated as previously described²⁴. Chromosomes were used immediately or frozen in N₂ and stored at -70 °C. For analysis of proteins, chromosomes were boiled in sample buffer and run on 7% SDS-polyacrylamide gels. Gels were either stained with Coomassie blue or blotted to nitrocellulose and probed with C-1 antibodies as described in Fig. 2. For immunofluorescence staining, a sample of the chromosome fraction was fixed with 5 mM ethylene glycol-bis(succinic acid N-hydroxysuccinimide ester) (EGS) in 80 mM PIPES, pH 6.8, 1 mM EGTA, and 1 mM MgCl₂, centrifuged onto a glass coverslip through a 40% glycerol cushion followed by post-fixation in MeOH at -20 °C.

specific antibodies (R-1 antibodies) that detected dynein on immunoblots of ATP extract fractions, whole HeLa cell protein, and whole PtK₁ cell protein (Fig. 2, lanes 4–6). These antibodies recognized both UV-cleavage fragments and the intact heavy chain (Fig. 2, lane 10), indicating their specificity for dynein heavy-chain polypeptides. Antibodies affinity purified against the heavier UV-cleavage fragment (HUV) recognized only this fragment and the intact heavy chain on immunoblots (Fig. 2, lane 11).

Antibodies directed against the 79K intermediate chain subunit of HeLa cytoplasmic dynein were raised in a chicken, also by injection of SDS-polyacrylamide gel bands. On affinity purification these antibodies (C-1 antibodies) reacted specifically with 79K polypeptides on immunoblots of ATP extract fractions, whole HeLa cell or whole PtK₁ cell protein (Fig. 2, lanes 7–9).

The affinity-purified antibodies were used for immunolocalization of cytoplasmic dynein in PtK₁ and HeLa cells (Fig. 3). Cells were either lysed with detergent and fixed in aldehyde, or fixed directly in cold MeOH. Both cell types showed similar distributions of dynein when labelled with either R-1 or C-1 antibodies. Cells lysed with detergent produced clearer images of anti-dynein staining of mitotic spindles, presumably as a result of the removal of solubilized dynein antigens.

Interphase cells stained throughout the cytoplasm with a granular pattern superimposed on a uniform background. The labelling was weaker at the cell periphery, but no intranuclear signal was detected (Fig. 3*a*). Prophase cells showed centrosome staining along with bright spots in the nuclear volume (Fig. 3*c*). These spotted regions were either within or very near the kinetochores; when cells were double-labelled with both anti-dynein antibodies and anti-kinetochore CREST serum²¹, the dynein staining in mitotic cells was found to co-localize precisely with kinetochores (Fig. 4*a, b*). At prometaphase, the dynein antibodies also stained fibres emanating from the spindle poles (Fig. 3*e*). In cells whose chromosomes appeared to have attached to the spindle microtubules, the staining became concentrated between the poles and the kinetochores, with a decreased staining of the 'astral' microtubules (Fig. 3*g*). Staining of spindle fibres was strong in metaphase and anaphase cells (Fig. 3*g, i*), with less intense staining in the interzone region between the separating chromosomes. By late anaphase, staining of all mitotic structures had decreased, and cells in cytokinesis showed an interphase pattern.

To confirm the localization of dynein to the kinetochore region, chromosomes isolated from mitotic HeLa cells were immunostained with the R-1 and C-1 antibodies. Both antibodies localized to the kinetochores, with the C-1 antibodies producing a stronger signal (Fig. 4*c, d*). When tested on immunoblots, C-1 antibodies stained only a 79K component of the chromosomal protein (Fig. 4*e*, lane 2). The R-1 antibodies reacted faintly with a 420K band in the chromosomal proteins, correlating with the weak immunofluorescence signal (data not shown).

The two antibodies used in this study were raised in different host organisms immunized with different subunits of cytoplasmic dynein isolated from different sources. Nevertheless, HeLa and PtK₁ cells fixed by two different methods produced similar staining patterns with both antibodies. These data indicate that cytoplasmic dynein is localized to kinetochores and other spindle structures, where it may have a role in the pole-directed movements of chromosomes during prometaphase². It could also contribute to the motion in anaphase A^{1,3,5}, either as a motor if ATP is present, or as a coupling to the microtubule lattice in the absence of ATP.

Note added in proof: See also the paper in this issue by Steuer *et al.*²⁵. □

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Localization of cytoplasmic dynein to mitotic spindles and kinetochores

E. R. Steuer*, L. Wordeman†, T. A. Schroer‡
& M. P. Sheetz*

* Department of Cell Biology and Physiology, Washington University
School of Medicine, Box 8228, 660 S Euclid Ave, St Louis,
Missouri 63110, USA

† Department of Pharmacology, University of California, San Francisco,
California 94143, USA

‡ Department of Biology, Johns Hopkins University, Baltimore,
Maryland 21218, USA

WHAT is the origin of the forces generating chromosome and spindle movements in mitosis? Both microtubule dynamics¹ and microtubule-dependent motors² have been proposed as the source of these motor forces. Cytoplasmic dynein and kinesin are two soluble proteins³⁻⁶ that power membranous organelle movements on microtubules^{7,8}. Kinesin directs movement of organelles to the 'plus' end of microtubules, and is found at the mitotic spindle in sea urchin embryos⁶, but not in mammalian cells⁹. Cytoplasmic dynein translocates organelles to the 'minus' end of microtubules, and is composed of two heavy chains and several light chains^{3,4}. We report here that monoclonal antibodies to two of these subunits and to another polypeptide that associates with dynein localize the protein to the mitotic spindle and to the kinetochores of isolated chromosomes, suggesting that cytoplasmic dynein is important in powering movements of the spindle and chromosomes in dividing cells.

Two monoclonal antibodies (440.1 and 440.4) against the heavy chain of chick brain cytoplasmic dynein react with a

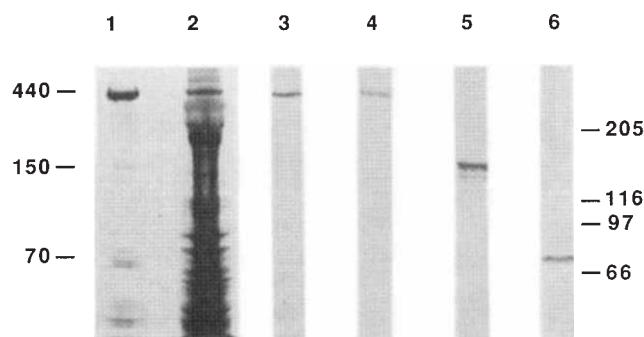


FIG. 1 Lanes 1 and 2, Coomassie blue-stained SDS-PAGE of chick brain cytoplasmic dynein heavy chain and its subunits^{3,4} purified by velocity sedimentation through sucrose (lane 1) and a high speed supernatant of CEF cells (lane 2). Lanes 3-6, immunoblots of the CEF supernatant probed with monoclonal antibodies 440.1 (lane 3), 440.4 (lane 4), 150.1 (lane 5) and 70.1 (lane 6). Immunoblots with 70.1 always appear as a triplet on purified preparations. The positions of the M_r standards are indicated on the right (in K), those of the dynein heavy chain (HC) and associated polypeptides (150s and 70s) are on the left.

METHODS. Cytoplasmic dynein was prepared as described in Fig. 2; CEF supernatant was prepared as described previously for S3 supernatant⁸. Monoclonal antibodies were prepared by injecting 4-6-week-old female BALB/c mice with 50 μ g cytoplasmic dynein prepared as described below and mixed with RIBI adjuvant (RIBI Immunochemicals). Antigen was injected intraperitoneally every two weeks for a total of four injections. Mice were injected intravenously with 50 μ g antigen (without adjuvant) dialysed against PBS 3 days before fusion of their spleen cells with mouse myeloma clone P3Ag 8.6.5.3. Hybridomas were screened by ELISA and positive clones were further characterized by immunoblotting. Immunoblots were incubated with hybridoma cell supernatants diluted 1:1 (lanes 3-5) or 1:5 (lane 6) and visualized with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Sigma).

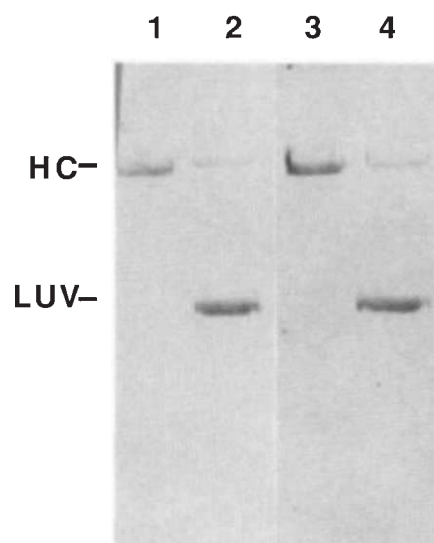


FIG. 2 Immunoblots of cytoplasmic dynein, intact (lanes 1, 3) and ultraviolet-vanadate cleaved (lanes 2, 4), stained with antibodies 440.1 (lanes 1, 2) and 440.4 (lanes 3, 4). The positions of the cytoplasmic dynein heavy chain (HC) and lower ultraviolet-vanadate fragment (LUV) are shown on the right. **METHODS.** Cytoplasmic dynein was purified by velocity sedimentation through sucrose and ultraviolet-cleaved as described previously⁸, except that the microtubule affinity step included 1 mM AMPPNP, the release conditions were modified to contain 2 mM ATP and 200 μ M Na_3VO_4 , and all buffers contained 0.9 M glycerol. Taxol was a gift from Matthew Suffness of the National Cancer Institute. The isolation of the monoclonal antibodies is described in Fig. 1 legend.

single band of relative molecular mass 440,000 (M_r 440K) in purified dynein preparations and in crude cell supernatants of chick fibroblast cells, as shown by immunoblots (Fig. 1). In addition, cleavage of the heavy chain into two fragments of $\approx$ 200K by exposure to ultraviolet light in the presence of ATP and vanadate^{3,4,10,11} demonstrates that the two antibodies react exclusively with the lower M_r fragment (Fig. 2). The monoclonal antibodies against the $\sim$ 150K dynein-associated polypeptide doublet (150.1) and the triplet of $\sim$ 70K light chains (70.1) each react specifically with bands of the appropriate M_r in immunoblots of cell supernatants of chick embryo fibroblasts (CEF) (Fig. 1). No other bands are seen in immunoblots of whole CEF cell lysates.

In intact mitotic CEF cells, the staining of the spindle with any of the anti-dynein antibodies is clearly visible through diffuse cytoplasmic staining. Detergent extraction of the cells in the presence of a microtubule-stabilizing buffer before fixation preserves spindle staining but markedly reduces cytoplasmic staining (Fig. 3), suggesting that dynein is present on the spindle during mitosis and is not there fortuitously as a result of fixation of whole cells.

Interphase cells show diffuse punctate staining throughout the cytoplasm with all the anti-dynein antibodies, consistent with observations that cytoplasmic dynein is a soluble protein in cytosolic extracts of cells^{3,4,8}. After detergent extraction, the staining is much fainter but still generally diffuse with punctate regions (Fig. 3). The staining in intact interphase cells does not resemble a microtubule pattern, although in extracted cells the punctate staining areas are occasionally aligned along microtubules, further supporting the view that cytoplasmic dynein is a soluble motor protein in interphase cells *in vivo*.

Throughout metaphase and anaphase, CEF cells show strong anti-dynein staining near the polar and kinetochore microtubules and the spindle poles (Fig. 3). During prometaphase, staining of the organizing spindle is weak, but there is bright, punctate staining of the chromosomes, suggestive of kinetochore

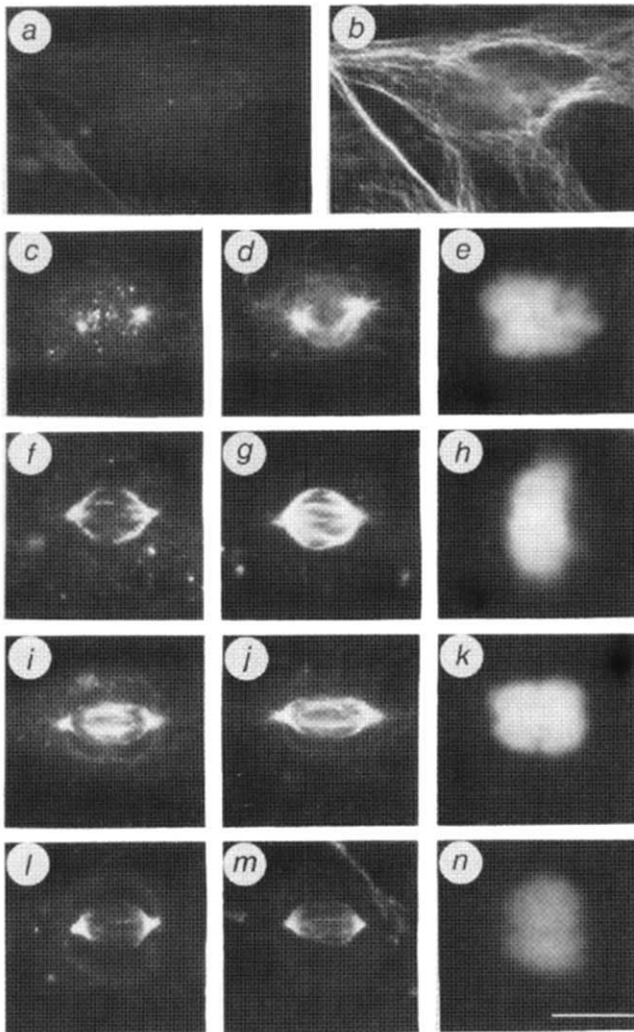


FIG. 3 Localization of cytoplasmic dynein to the mitotic spindle in dividing CEF cells extracted with a detergent-containing, microtubule-stabilizing buffer (rows 1–4) or microtubule-destabilizing buffer (row 5) before fixation, also stained for tubulin and chromosomes. Row 1, interphase CEF cell; 2, prometaphase CEF cell; 3, metaphase CEF cell; 4, anaphase CEF cell; and 5, metaphase CEF cell, extracted before fixation under conditions designed to depolymerize all but the kinetochore microtubules. Cells were stained for dynein. (a, c, f, i, l), Tubulin (b, d, g, j, m) and chromosomes (e, h, k, n). Bar, 10 μ m.

METHODS. Primary CEFs were prepared from 11-day-old chick embryos and grown in 10-cm dishes. Secondary CEFs were plated on coverslips 18–24 h before use. For microtubule-stabilizing conditions (a–k), coverslips were dipped in PBS and then PEEM (0.1 M PIPES, pH 6.9, 1 mM MgSO_4 , 0.1 mM EDTA and 2 mM EGTA) and then placed in dishes containing PEEM plus 0.5% Triton X-100 (Pierce), 1 mM dithiothreitol and a mixture of protease inhibitors described previously⁸, all at 37 °C. After 30 s, coverslips were blotted dry and plunged immediately into –80 °C methanol and 1.0% paraformaldehyde and placed into a freezer at –20 °C for 30 min. For microtubule-destabilizing conditions (l–n), cells were extracted at 4 °C for 5 min in a buffer containing 0.5% Triton X-100 and 80 μ M CaCl_2 as described previously¹² and fixed as described above. Coverslips were rehydrated in TTBS (0.3 M NaCl, 20 mM Tris (pH 7.5), 0.01% NaN_3 , and 0.05% Tween-20). Coverslips were rinsed again in TTBS and blocked overnight in 10% normal goat serum in TTBS. Blocked coverslips were rinsed in PBS and incubated for 1 h at 37 °C with a 50:50 mixture of 440.1 and 440.4 cell supernatants, rinsed three times in TTBS, 10 min each rinse, incubated for 1 h at 37 °C with a 1:50 dilution of fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG (Boehringer) in 10% normal goat serum in PBS, rinsed as before, incubated for 1 h at 37 °C with a 1:50 dilution of a rat anti-tubulin monoclonal YOL1/34 (ref. 19) (Accurate Chemicals) in PBS, rinsed as before, incubated for 1 h at 37 °C with a 1:50 dilution of rhodamine-labelled goat anti-rat IgG (Cappel) in 10% normal goat serum in PBS and rinsed as before. The first of the last three TTBS rinses contained 0.05 μ g ml^{–1} bis-benzimide (Hoechst 33258). The goat anti-mouse and goat anti-rat IgGs were each preadsorbed against IgGs from rat and mouse, respectively. Control slides showed no nonspecific cell staining, and no visible cross-reactivity remained in the second antibodies after preadsorption. Coverslips were dipped in 50% (v/v) glycerol in PBS, mounted in the same solution plus 1% *N*-propyl-gallate and observed under a Zeiss Axioplan microscope equipped with a Plan Neofluar 100 $\times$ (NA 1.3) objective lens. Micrographs were taken with TMAX 400 ASA black and white print film (Kodak).

staining. Cells in telophase show staining of the spindle poles and frequently the midbody. In immunofluorescence experiments with the other antibodies, we consistently observe an identical staining pattern consisting of the central portion of the mitotic spindle.

To determine if the staining of the spindle was on kinetochore or non-kinetochore microtubules, we extracted CEF cells with 0.5% Triton X-100 at 4 °C in the presence of 80 μ M CaCl_2 to depolymerize all but the kinetochore microtubules¹². CEF cells treated in this manner still show intense spindle staining with

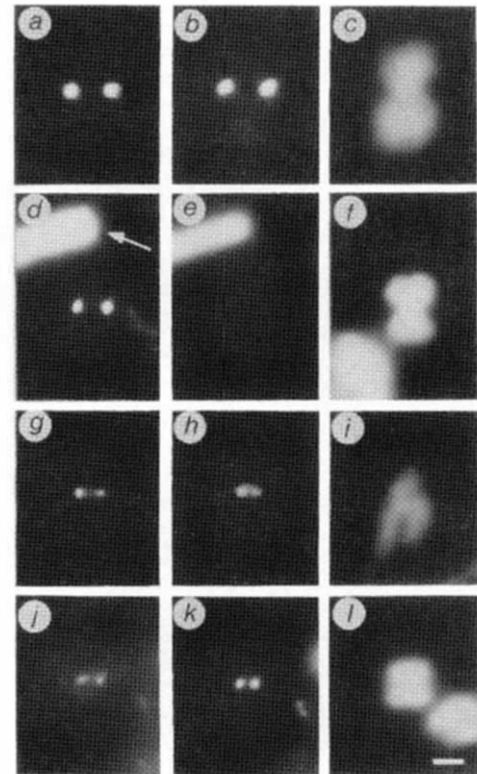


FIG. 4 Localization of cytoplasmic dynein to kinetochores in isolated CHO (a–f) and CEF (g–l) chromosomes. Isolated CHO chromosomes from colcemid-blocked CHO cells double-labelled with 70.1 (a) and anti-tubulin (b). Vinblastine-blocked CHO chromosomes double-labelled with 70.1 (d) and anti-tubulin (e) show that dynein can be localized to CHO kinetochores even in the absence of visible tubulin staining (arrow; tubulin-containing vinblastine paracrystal). The vinblastine paracrystal stains with both anti-dynein and anti-tubulin antibodies. Anti-microtubule-associated protein (MAP) staining of vinblastine paracrystals has been observed previously^{20,21}. In addition, vinblastine paracrystals have been observed to move on microtubules *in vitro* in the presence of ATP (A. A. Hyman, personal communication). Chromosomes isolated from mitotic CEFs were labelled with 70.1 (g) and 440.4 (j) and double-labelled with human CREST sera (h, k). DNA was visualized with Hoechst 33342 (Sigma), (c, f, i, l). Bar, 1.5 μ m.

METHODS. CHO chromosomes were isolated from vinblastine-arrested mitotic cells as described previously²², except that the lysis buffer was slightly modified to 10 mM Pipes, 5 mM MgCl_2 , 1 mM EGTA, pH 6.9 (PME) plus 0.1% digitonin. These chromosomes had little or no endogenous tubulin associated with the kinetochore. Chromosomes prepared in this way from CHO cells that were incubated 16 h with 0.1 μ g ml^{–1} colcemid (Sigma), instead of vinblastine, have kinetochore-associated tubulin staining after isolation. CEF chromosomes were prepared using a procedure slightly modified from that described above. Mitotic CEFs were collected by mitotic shake-off from unsynchronized log-phase primary CEF culture provided by I. Tuet (University of California, San Francisco). The fibroblasts were gently lysed with one pass through a 18-gauge needle and spun through a 30% glycerol cushion in PME. The gentler technique was necessary to preserve anti-dynein staining of these chromosomes. Isolated chromosomes were fixed in 0.5% paraformaldehyde (Ted Pella, Inc.) for 10 min at 20 °C, pelleted onto coverslips and post-fixed in –20 °C methanol. Coverslips were blocked for 5 min. in 1% BSA (20 °C) and then incubated for 30 min at room temperature with 440.4 (full strength) or 70.1 (diluted 1:5 in 1% BSA) rinsed in TTBS, and double labelled with either rat anti-tubulin monoclonal antibody YOL1/34 (ref. 19) or CREST anti-kinetochore sera (provided by F. McKeon, Harvard Medical School) rinsed as before and mounted in 0.02% *p*-diamino-benzene (Sigma) in glycerol–Tris pH 8.2. Coverslips were observed with an Olympus SPlan Apo 60 $\times$ objective lens mounted on a Zeiss Photoscope III. Micrographs were taken with hypered Technical Pan film (Kodak) and developed in HC110, dilution D.

cytoplasmic dynein antibodies (Fig. 3). This result strongly suggests that dynein is localized on kinetochore microtubules during metaphase and anaphase, but does not exclude the possibility that it is also localized on polar microtubules.

To demonstrate that our results were not limited to CEF cells, we used several of our antibodies to stain PtK1 cells, a marsupial (rat kangaroo) kidney cell line. The monoclonal antibody against the 70K polypeptides (70.1) and the goat polyclonal antiserum cross-react with mammalian cytoplasmic dynein (data not shown). The similarity of the spindle staining between the fibroblasts and the PtK1 cells (data not shown) suggests that cytoplasmic dynein localization to the spindle is not unique to chick fibroblasts.

Because cytoplasmic dynein may be involved in chromosome movement, we stained isolated chromosomes from CEF cells with monoclonals 440.1, 440.4, 150.1 and 70.1, finding that the staining co-localizes with kinetochore staining, using CREST serum¹³. Furthermore, monoclonal 70.1 co-localizes with kinetochores in whole CHO cells as well as in isolated chromosomes (Fig. 4) and HeLa cells (data not shown). We also found that monoclonal 440.4 cross-reacts with kinetochores in isolated HeLa chromosomes, although not with isolated CHO chromosomes (data not shown). Finally, anti-dynein staining of isolated CHO chromosomes is not dependent on the association of tubulin with the kinetochore (Fig. 4). We conclude that cytoplasmic dynein is at the kinetochore of chromosomes in dividing cells.

Although antibodies to axonemal dynein from sea urchin bind to the spindle in sea urchin eggs¹⁴⁻¹⁶, localization of cytoplasmic dynein to the spindle has not been reported previously. Immunolocalization of cytoplasmic dynein to the mitotic spindle in both vertebrate cells (see also Pfarr *et al.*, this issue¹⁷) and the nematode *Caenorhabditis elegans* (R. J. Lye *et al.*, personal communication), raises the possibility that the enzyme plays a part in mitosis. These light-microscope studies do not identify the site of force generation, but indicate that in mitotic cells, cytoplasmic dynein is bound to kinetochore microtubules and to the kinetochore itself. Although cytoplasmic dynein is not localized exclusively on the kinetochore and kinetochore microtubules, it might be involved in the minus-end-directed movement of chromosomes during anaphase A and other stages of mitosis¹⁸. Presumably, to exert force on the chromosome, cytoplasmic dynein would be bound by one end to the kinetochore and by the other to the kinetochore microtubule(s).

It is apparent that there is a redistribution of cytoplasmic dynein at the onset of mitosis. The differences in the staining intensity between extracted interphase and mitotic cells supports the notion that dynein associates with a specific set of microtubules during mitosis, particularly those in the central portion of the spindle. One explanation for the regulation of this interaction could be a cell-cycle-dependent post-translational modification of cytoplasmic dynein or an activation of a dynein-binding protein that alters the affinity of dynein for spindle microtubules. □

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The gain of two chloroplast tRNA introns marks the green algal ancestors of land plants

J. R. Manhart & J. D. Palmer*

Department of Biology, Texas A&M University, College Station, Texas 77843, USA; * Department of Biology, Indiana University, Bloomington, Indiana 47405, USA

THE relationship of green algae to land plants has greatly interested botanists for more than a century. In recent years, several characters, particularly ultrastructural ones, have been used to define a green algal group (Charophyceae) from which land plants are thought to have arisen (refs 1-3, but see ref. 4). Here we provide the first molecular genetic evidence in support of the charophycean origin of land plants. Group II introns have previously been found in both the tRNA^{Ala} and tRNA^{Ile} genes of all land plant chloroplast DNAs examined, whereas all algae and eubacteria examined have uninterrupted genes⁵⁻¹⁹. The distribution of these introns in *Coleochaete*, *Nitella* and *Spirogyra*, members of the Charophyceae, confirms that these taxa are part of the lineage that gave rise to land plants. Furthermore, the intron data place *Coleochaete* and *Nitella* closer to land plants than *Spirogyra*. These introns were most probably acquired by the chloroplast genome more than 400-500 million years ago, the time of land plant origin²⁰.

The tRNA^{Ala} and tRNA^{Ile} genes are typically located in the transcribed spacer between the 16S and 23S rRNA genes in chloroplasts and bacteria⁵⁻¹⁹. The complete nucleotide sequence of a 2,305-base pair (bp) region containing this spacer, 183 bp of the 3' end of the 16S rRNA and 538 bp of the 5' end of the 23S rRNA genes was determined for *Coleochaete orbicularis* chloroplast DNA. Members of the genus *Coleochaete*, of all the green algae, have been placed closest to the land plant lineage^{2,3}.

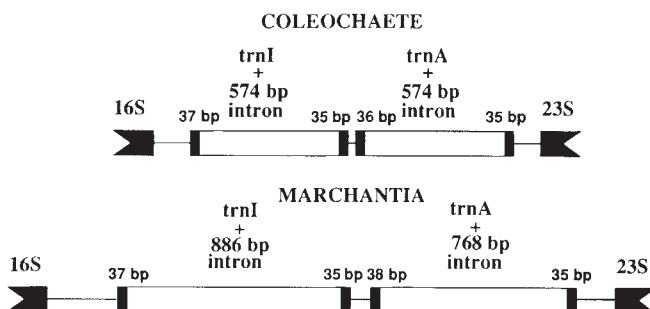


FIG. 1 Comparison of rRNA spacer regions of *Coleochaete orbicularis* and *Marchantia polymorpha*¹⁴ chloroplast DNAs. Nucleotide sequence of 2,305 bp of a 3.2-kb *Hind*III fragment from *Coleochaete orbicularis* chloroplast DNA cloned into pBluescript (pBS, Stratagene) was determined using a combination of subclones and synthetic primers. Sequencing reactions with both dGTP and dTTP (Sequenase) produced a series of overlapping sequences using double-stranded DNA. The entire sequence has been deposited with GenBank and is also available from the authors.

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The organization of this spacer is quite similar to that found in *Marchantia polymorpha*¹⁴ and other land plants (Fig. 1). The main difference is that the intergenic spacers and the introns themselves are all smaller in *Coleochaete* than in *Marchantia*. A comparison of the tRNA exons and introns in *Coleochaete* and *Marchantia* (Fig. 2a) reveals that the introns are in exactly the same position in both genes. In addition, the regions of the introns adjacent to the exons have sequence similarities of 66.9–75% (Fig. 2a). These similarities in position and sequence indicate that these two tRNA introns of *Coleochaete* and *Marchantia* had a common origin.

Sufficient portions of these tRNA genes were sequenced in *Nitella axillaris* and *Spirogyra maxima* to determine whether

introns are present in them as well (Fig. 2b–e). These taxa are also members of the Charophyceae, but are sometimes placed more distantly from the land plant lineage than *Coleochaete*^{2,3}. Introns are present in the tRNA^{Ala} and tRNA^{Ile} genes in *Nitella* and the tRNA^{Ala} gene in *Spirogyra* (Fig. 2b–d). The introns again occur in precisely the same position as in *Marchantia* and show sequence similarities of 63.5–71.8% to the *Marchantia* introns (Fig. 2b–d). The tRNA^{Ile} gene in *Spirogyra* does not contain an intron (Fig. 2e).

Assessment of the direction of evolution of these introns based on parsimony strongly suggests that they were acquired in the Charophyceae and were absent in their ancestors. The most parsimonious tree (Fig. 3a) postulates a single gain of each

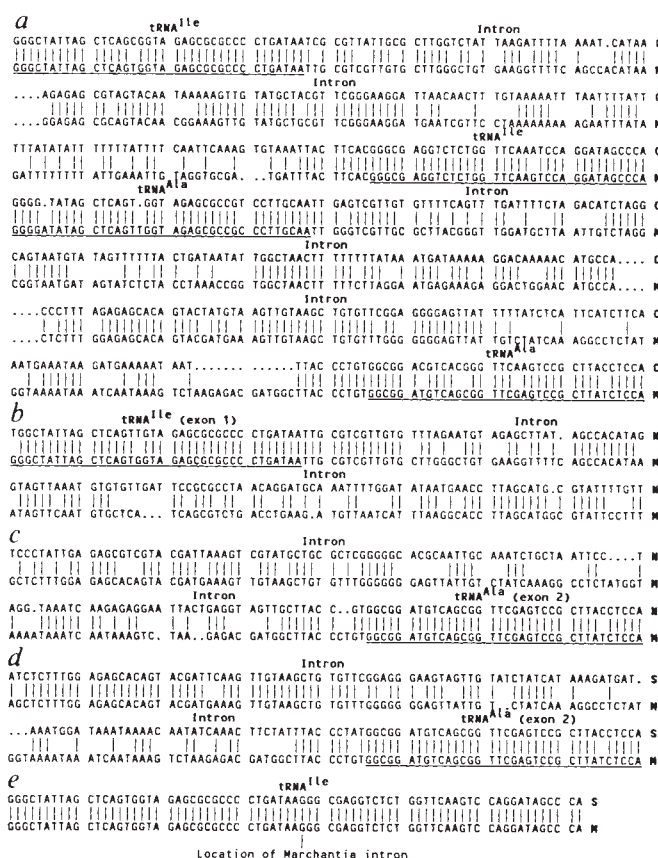


FIG. 2 a, Sequence alignment of tRNA^{Ile} and tRNA^{Ala} exons and adjacent portions of introns from *Coleochaete* (C) and *Marchantia* (M). Sequence similarities of intron regions are 75% and 66.9% for tRNA^{Ile}; 66.9% and 69.4% for tRNA^{Ala}. b, Sequence alignment of *Marchantia* (M) tRNA^{Ile} exon 1 and adjacent portion of intron with partial sequence of *Nitella axillaris* (N) 0.9-kb *EcoRI* chloroplast DNA pBS clone. Sequence similarity of intron region is 71.8%. c, Sequence alignment of *Marchantia* tRNA^{Ala} exon 2 and adjacent intron with partial sequence of *Nitella* 3.3-kb *EcoRI* chloroplast DNA pBS clone. Sequence similarity of intron region is 63.5%. d, Sequence alignment of *Marchantia* tRNA^{Ala} exon 2 and adjacent portion of intron with partial sequence of *Spirogyra* (S) 9.0-kb *PstI* chloroplast DNA pBS clone. Sequence similarity of intron region is 68.9%. e, Sequence alignment of tRNA^{Ile} from *Spirogyra* 15-kb *Clal* chloroplast DNA pBS clone and exons 1 and 2 from *Marchantia*. There is no intron in this gene in *Spirogyra* chloroplast DNA. The sequences in this report have been deposited with GenBank and are also available from the authors.

METHODS. *Nitella* and *Spirogyra* sequences were obtained using synthetic primers to conserved regions of the 16S and 23S rRNA genes, exon 1 of tRNA^{Ile} and exon 2 of tRNA^{Ala}. Alignments were done with programs from the University of Wisconsin Genetics Computer Group Sequence Analysis Software (Version 6.0)³¹ and visually. The regions of the introns adjacent to the exons were the only ones that displayed sequence similarities >50%.

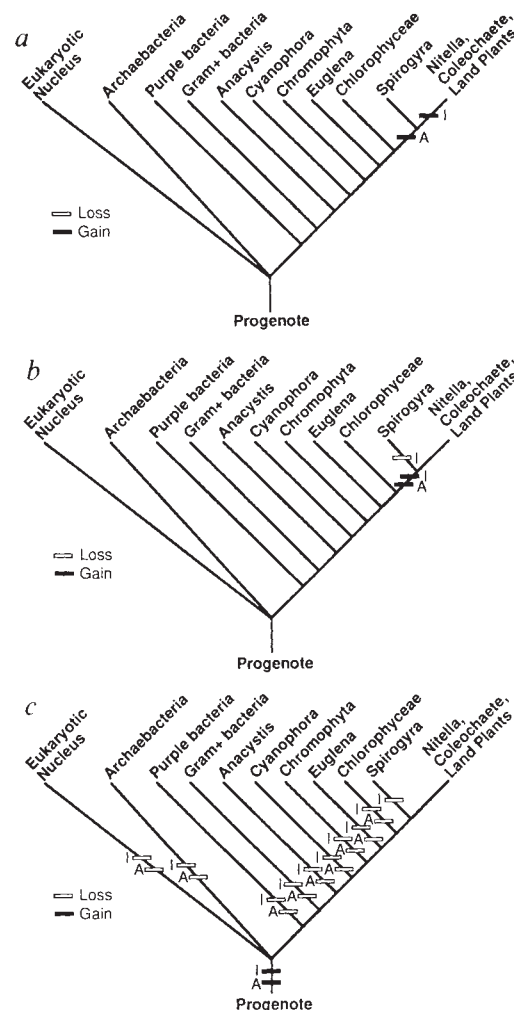


FIG. 3 Cladograms based on distribution of tRNA^{Ile} and tRNA^{Ala} introns (Table 1) with scenarios a, b, c for observed presence/absence of introns. Branching pattern of the taxa excluding *Spirogyra*, *Nitella*, *Coleochaete*, and land plants is based on phylogenetic trees produced from phenetic characters^{2,3} and 16S rRNA sequences^{26,27}. The exact placement of the hypothetical progenote is unknown although recent evidence suggests that the root lies between the trifurcation and the eubacteria³². Taxa included in each clade: eukaryotic nucleus tRNA^{Ala} 4 spp. (ref. 5), tRNA^{Ile}, 4 spp. (ref. 5); archaeobacteria tRNA^{Ala}, 9 spp. (ref. 5), tRNA^{Ile}, *Halobacterium volcanii* (R. Gupta, personal communication); purple bacteria, 3 spp. (ref. 5); Gram-positive bacteria, 3 spp. (ref. 5); cyanobacteria, *Anacystis nidulans*⁶; Chromophyta, *Pylaiella littoralis*⁷, *Olisthodiscus luteus*⁸, *Cryptomonas* sp. (S. Douglas, personal communication); *Euglena gracilis*⁹ and *Cyanophora paradoxa*¹⁰ were not placed in a specific taxonomic group because of uncertainties about their phylogenetic affinities; Chlorophyceae, *Chlamydomonas reinhardtii*¹¹, *Chlorella ellipsoidea*^{12,13}; land plants, *Marchantia polymorpha*¹⁴, *Nicotiana tabacum*¹⁵, *Pisum sativum*¹⁶, *Glycine max*¹⁷, *Spinacia oleracea*¹⁸, *Zea mays*¹⁹.

intron, with the tRNA^{Ala} gene acquiring its intron in the common ancestor of land plants and Charophytes between 400 and more than 500 million years ago, and the tRNA^{Ile} gene its intron after the divergence of *Spirogyra*. A tree one step longer postulates the gain of both introns in the Charophyte-land plant ancestor and the subsequent loss of the tRNA^{Ile} intron in the lineage leading to *Spirogyra* (Fig. 3b).

An alternative hypothesis, which treats introns as primitive, assumes that introns were present in the long-extinct progenote from which all extant organisms descended. It is not possible to test this hypothesis directly and the arguments pro and con have focused both on mechanistic and phylogenetic models of molecular processes²¹⁻²⁵. A phylogenetic analysis of the tRNA introns allows an opportunity for limited testing of the introns-as-primitive hypothesis. It is clear from analyses of rRNA sequences that chloroplasts are derived from within the cyanobacteria²⁶, which themselves are relatively derived members of the eubacterial lineage²⁷. If all introns are primitive we are forced to assume that the tRNA introns were retained throughout all the major branchings of eubacterial evolution²⁷ leading to cyanobacteria and, subsequently, to chloroplasts. Therefore, the acceptance of an all-inclusive introns-as-primitive hypothesis forces a violation of one of its important tenets, which is that bacteria do not have introns because of an early loss of introns in these lineages²⁸. Other serious difficulties with the assumption that these tRNA introns are primitive become obvious with formal phylogenetic analysis. Figure 3c was constructed by assuming that the tRNA introns were primitively present in the progenote and subsequently lost in all lineages lacking them. According to this scenario, the introns were selectively retained in Charophytes and land plants. The number of parallel losses postulated by this tree is so high (at least 19) that it cannot be seriously considered.

Gross changes in chloroplast genomes such as intron gains and losses, gene losses, and inversions are proving valuable in testing phylogenetic hypotheses in green plants^{29,30}. In the example presented here, the most parsimonious tree drawn from the distribution of the two tRNA genes and our present understanding of bacterial and algal relationships assumes them to be derived. This tree places the Charophyceae with land plants and *Nitella* and *Coleochaete* closer to land plants than *Spirogyra* (Fig. 3a). A survey of representatives of all major groups of green algae for the distribution of these two introns and the additional seventeen introns found in land plant chloroplast DNAs^{14,15} may help further delimit land plant lineage. □

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Human U2 snRNA can function in pre-mRNA splicing in yeast

E. O. Shuster* & C. Guthrie

Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143, USA

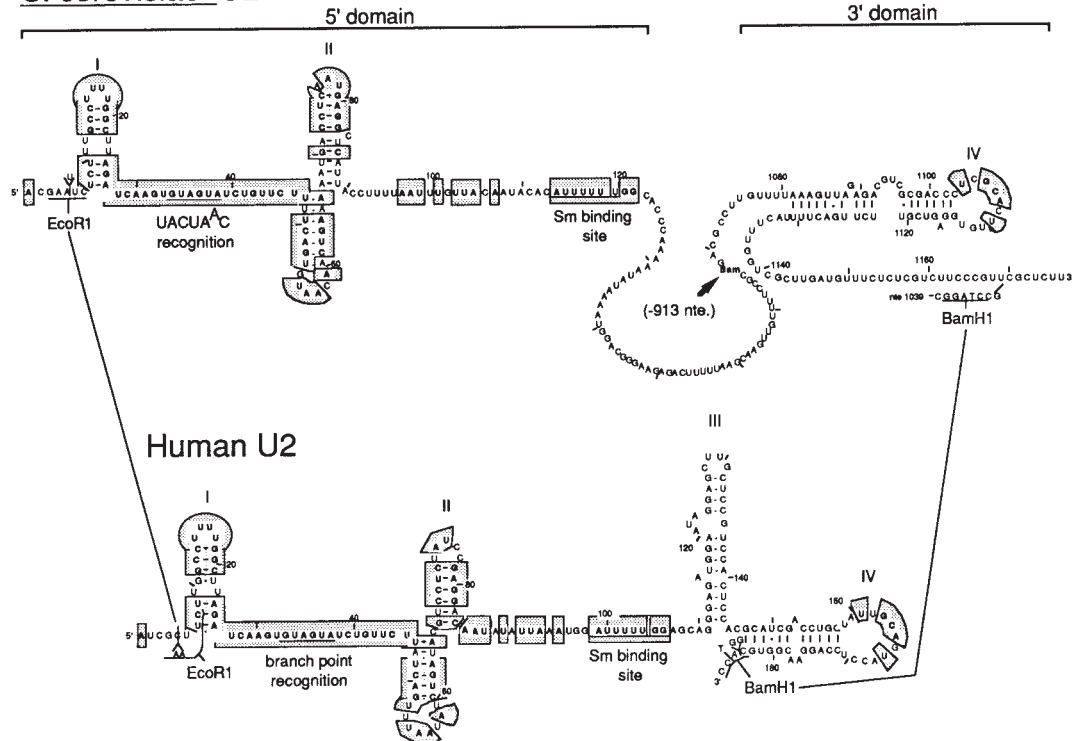
THE removal of introns from messenger RNA precursors requires five small nuclear RNAs (snRNAs), contained within ribonucleoprotein particles (snRNPs), which complex with the pre-mRNA and other associated factors to form the spliceosome (reviewed in refs 1-3). In both yeast and mammals, the U2 snRNA base pairs with sequences surrounding the site of lariat formation⁴⁻⁹. Binding of U2 snRNP to the highly degenerate branchpoint sequence in mammalian introns is absolutely dependent on an auxiliary protein, U2AF, which recognizes a polypyrimidine stretch adjacent to the 3' splice site¹⁰. The absence of this sequence motif in yeast introns has strengthened arguments that the two systems are fundamentally different^{10,11}. Deletion analyses of the yeast U2 gene have confirmed that the highly conserved 5' domain is essential, although the adjacent ~950 nucleotides can be deleted without any phenotypic consequence^{12,13}. A 3'-terminal domain of ~100 nucleotides is also required for wild-type growth rates; the highly conserved terminal loop within this domain (loop IV) may provide specific binding contacts for two U2-specific snRNP proteins¹³⁻¹⁵. We have replaced the single copy yeast U2 (yU2) gene with human U2 (hU2), expecting that weak or no complementation would provide an assay for cloning additional splicing factors, such as U2AF. We report here that hU2 can complement the yeast deletion with surprising efficiency. The interactions governing spliceosome assembly and intron recognition are thus more conserved than previously suspected. Paradoxically, the conserved loop IV sequence is dispensable in yeast.

To facilitate proper expression of the hU2 gene in yeast, it was placed in the context of the promoter and terminator of the yU2 gene (*SNR20*; ref. 16, or *LSR1*; ref. 11) as illustrated in Fig. 1. The resulting construct was assayed for function using the plasmid shuffle method (ref. 17; Fig. 1). No growth defect was observed in cells containing both yeast and human U2 genes (data not shown), indicating that the hU2 RNA does not have a dominant negative effect. Notably, growth of cells containing only hU2 was surprisingly robust at 30 °C (only 1.5 times slower than yU2 controls), although these cells failed to form colonies at either 18 or 37 °C (Fig. 2). Temperature shift experiments revealed that the cold-sensitive and temperature-sensitive defects were not shown immediately on transfer to the restrictive temperature; rather, growth rate gradually slowed (with respect to yU2 controls) over 10-24 h (data not shown). This implies that the primary defect caused by hU2 is in the assembly and/or stability of splicing complexes, rather than in splicing function. The expectation that these cells did, in fact, lack yU2 RNA was confirmed by RNA sequencing (data not shown) and Northern analysis (Fig. 3, hU2a/b). Although no RNA of the size expected for full length yU2 was detected, several species ranging from 15-30 nucleotides larger than that expected for hU2 were

* Present address: Department of Food Science, University of California, Davis, California 95616, USA.

FIG. 1 Expression of human U2 gene in yeast. The *S. cerevisiae* yU2 sequence is that of the yU2-107 allele which lacks 893 nucleotides of non-essential coding sequence (*snr20-107*, ref. 13). Numbering reflects position within the full-length yU2 gene. The bold arrow indicates the location of the *Bam*H1 linker at the junction between the 5' and 3' portions of the allele. The RNA structures have been modified from those shown previously^{12,13} in the light of recent data from M. Ares (personal communication). Boxes indicate nucleotides conserved between hU2 and yU2 (no regions of obvious similarity are missing from yU2-107). The 5' and 3' domains are as previously defined¹³. The conserved 5' termini contain two sequence motifs of known importance: (1) an Sm binding site required for the association with core snRNP proteins²¹; (2) the sequence which base pairs with the branchpoint region in the intron⁹. Oligonucleotide

S. cerevisiae U2



mutagenesis of single-stranded template prepared from *dut⁻, ung⁻* cells^{22,23} was used to introduce *Eco*RI and *Bam*HI restriction sites at the 5' and 3' termini of the human U2 gene (provided by M. Ares, ref. 24), placing the human gene within the context of the yU2 controlling elements. An *Eco*RI site was introduced into yU2-301, a yU2 allele which contains a *Bam*HI linker six nucleotides from the 3' end. The identical *Eco*RI mutation in yU2-6 was shown not to affect growth (this assay could not be carried out in yU2-301, since the 301 allele itself causes a severe growth defect¹³). The *Eco*RI/*Bam*HI fragment of yU2-301/RI was replaced with the hU2 *Eco*RI/*Bam*HI fragment. The resulting human U2 expression plasmid (pES150) consisted of a *Sall*/*Sma*I fragment containing the hU2 coding

region flanked by ~780 nucleotides of upstream and ~475 nucleotides of downstream yU2 sequence carried on a *TRP1, CEN4* vector (pSE358; ref. 13). The hU2 construct was transformed into strain ES223.5.1 (*MAT α , SNR20::LYS2, lys2, ura3, trp1, his3, ade2*, ref. 13) using the LiOAc method of Ito *et al.*²⁵ and the resulting strain (ES223.5.1/pES150) was assayed for growth in the presence and absence of a plasmid containing yU2 (pES15 [*SNR20, CEN4, URA3*] ref. 13) using the plasmid shuffle method¹⁷. Only cells capable of growth in the absence of the *URA3*-containing plasmid (pES15) will grow in the presence of the drug 5-fluoro-orotic acid (5-FOA). As this plasmid also carries the wild-type allele of yU2, cells will survive only if the human U2 gene can support cell division.

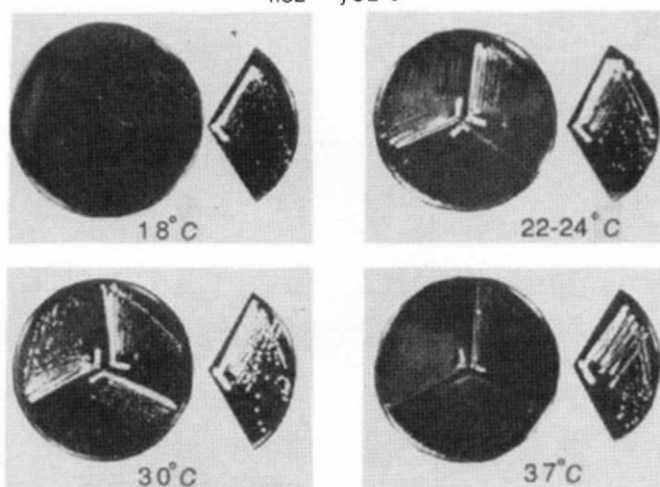
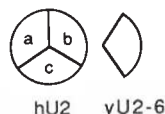
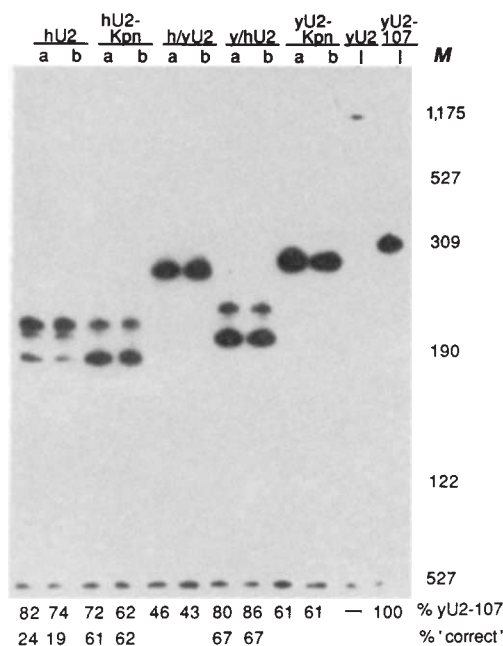


FIG. 2 Growth of hU2-containing cells. Three independent isolates of an hU2⁺, yU2⁻ strain (obtained as in Fig. 1) were assayed for growth on plates at 18 °C, room temperature (22–24 °C), 30 and 37 °C. For comparison, a strain containing yU2-6 was assayed in parallel (yU2-6 has no deleterious effect upon growth or RNA biogenesis, ref. 13). The photographs presented here were taken after four days of growth.

observed in addition to the predicted 190-nucleotide species. As length polymorphisms at the 5' end of hU2 were not detected in RNA sequencing reactions (data not shown), we assume that the larger species arise through defects in 3'-end formation and/or processing, as observed in cells carrying deletions within yU2 stem/loop IV (ref. 13). Intriguingly, the ratio of the 190-nucleotide ('correct') hU2 to the 220-nucleotide ('elongated') species decreased with prolonged incubation at either 18 or 37 °C (data not shown). The elongated hU2 molecules may be less able to form stable, functional complexes than are the correctly sized molecules.

To identify the region(s) of hU2 responsible for the growth defects, hybrid U2 genes were constructed and analysed *in vivo*. The 5' and 3' domains of hU2 and yU2-107 (a minimal yU2 allele; Fig. 1) were separated by the introduction of a *Kpn*I site just 3' of the Sm binding site and rejoined to form hybrid genes (h/yU2 and y/hU2) which retained 5' and 3' flanking sequences from yeast (Fig. 4; M. Ares, personal communication). Surprisingly, the *Kpn*I mutation alone improved the function of hU2 (Fig. 4). But, as strains containing either hybrid divided more rapidly, at all temperatures, than the hU2-Kpn strains (Fig. 4), the hU2 5' and 3' domains must also contribute to the observed temperature sensitivity. Similar observations of h/yU2 growth have been made by M. Ares and S. Seiwert (personal communication). Northern analysis revealed that defects in 3'-end formation were strictly correlated with the presence of the hU2 3' domain (Fig. 3). As predicted, differences in the ratio of correctly sized to elongated U2 roughly paralleled differences in growth



rate; that is, higher levels of correctly sized U2 (Fig. 3) were found in strains with faster growth rates (Fig. 4).

To dissect the role of the 3' domain further, we altered the most highly conserved nucleotides of loop IV in the context of yU2-6 (ref. 13). In the most extreme case, we replaced the 10-nucleotide loop with a 4-nucleotide 'tetra-loop' (UUCG, ref. 18). No significant effects on growth rate or RNA biogenesis (data not shown) were observed. When the tetra-loop mutation was placed in the context of the yU2-min1 allele, the smallest in our collection capable of supporting growth at wild-type rates (ref. 13), a modest decrease in growth was detected at 18, 23 and 37 °C (data not shown). A similar result was obtained with a 10-nucleotide substitution of loop IV (5'GAUACAGGUG3'), suggesting that both the sequence and the size of the conserved loop domain contribute to the maximal performance of the U2 RNA. As yeast U2 snRNAs with deletions of the terminal stem/loop can still grow, albeit at a severely reduced rate, this domain is not essential to splicing¹³. Additional support for this conclusion comes from yeast reconstitution experiments *in*

FIG. 3 Northern analysis of U2 RNAs. Samples (15 µg) of genomic RNA, prepared by the guanidinium thiocyanate method²⁶ from cells incubated at room temperature (22–24 °C), were run on a 6% acrylamide, 7 M urea gel, transferred to nylon membrane and probed with a 3' end-labelled oligonucleotide complementary to the highly conserved branchpoint recognition site (U2-L15, refs 27, 13). As a control for differences in the amount of RNA loaded, the gel was washed and re-probed with an oligonucleotide complementary to U4. Two independent isolates of each strain were examined, except for yU2-107 and yU2-WT. The relative amounts of U2 RNA in each strain (calculated from densitometric scans) are expressed as a percentage of the level observed in the yU2-107 strain. Where multiple U2 species are present, the percentage of correctly sized U2 (relative to the total level of U2 in the strain) is presented. The faint 527 nucleotide band is not derived from the yU2 gene and does not appear to be affected by alterations in yU2 (ref. 13). A darker exposure of this band (lower panel) was subjected to densitometric analysis and used to correct for differences in the amount of RNA loaded in each lane. As RNA species larger than ~700 nucleotides transferred poorly in the conditions used (data not shown), the amount of full-length yU2 cannot be quantitatively compared with the amounts of other U2 species.

*vitro*¹⁹ and microinjection experiments in *Xenopus*^{15,20} in which U2 transcripts lacking stem/loop IV supported splicing.

Interestingly, the mutant *Xenopus* U2 molecules cannot (stably) bind the U2 snRNP-specific proteins A' and B'' (ref. 15). Further experiments will be required to ascertain whether the yeast loop IV mutations prevent binding of the yeast analogues of A' and B'', implying that these proteins are not required for snRNP biogenesis or function *in vivo*. If binding is still observed, this would show that loop IV is not the main component of the protein-binding site.

The ability of human U2 to complement a lethal deletion of the yeast gene demonstrates that the human RNA can: (1) be expressed in the context of yeast U2 transcription signals; (2) be packaged into a stable snRNP particle; and (3) associate with the yeast U1, U4/U6 and U5 snRNPs to recognize intron signals and assemble a functional spliceosome. Given the substantial differences between the yeast and human RNAs in size, sequence and secondary structure (Fig. 1), this result can be interpreted as a successful outcome of a massive mutagenesis

FIG. 4 Structure of hybrid U2 snRNAs. *KpnI* sites were introduced by oligonucleotide mutagenesis^{22,23} into a non-conserved region just distal to the Sm sites of both yU2-107 and hU2 (see Fig. 1). Oligonucleotides HK (5'CTCCCTGGTACCAAAATCC3') and YK (5'ACATTTTTTGGTACCAAAA3') (base changes are underlined) were provided by M. Ares. Hybrid genes were constructed by exchanging the *SalI/KpnI* fragment of hU2-Kpn (containing the yeast U2 5' flanking sequence and the hU2 5' domain) with the analogous fragment of yU2-107/Kpn. Vector sequences (pSE358: *CEN4*, *TRP1*) remained unchanged. A schematic representation of each construct, with the approximate locations of pertinent restriction sites, is presented along with the estimated size of the resulting U2 snRNA and the nucleotide sequence of the junction region. Differences from wild type (hU2 or yU2, as appropriate; note that yU2-107 is identical to wild-type yU2 in this region) are indicated by bold letters. *KpnI* sites introduced by oligonucleotide mutagenesis are underlined. Each construct was introduced into strain ES223.5.1 and assayed for its ability to support growth at 18 °C, room temperature (22–24 °C), 30

Species	Predicted size	Sm site	Non-conserved region	Doubling time (h)				
				18°C	22–24°C	30°C	37°C	
yU2-WT	1,175	110' A CAUUUUUUUGGCACCCUU' 127		5.5	2.5	1.8	2.3	
hU2	188	98' G GAUUUUUUUGGAGCAGGG' 113		11.8	8.3	2.8	3.6	
hU2-Kpn	189	G GAUUUUUUUGGUAACAGGG		9.8	4.1	2.3	3.0	
yU2/hU2	203	A CAUUUUUUUGGUAACAGGG		8.1	3.1	1.9	2.6	
hU2/yU2	275	G GAUUUUUUUGGUAACCCUU		6.8	2.4	1.9	2.6	
yU2-107/Kpn	305	A CAUUUUUUUGGUAACCCUU		5.3	2.4	1.8	2.3	
yU2-107	305	110' A CAUUUUUUUGGCACCCUU' 127		5.3	2.4	1.8	2.2	

913 nte B = *Bam*HI site(s)
K = *KpnI* site R = *Eco*RI site

or 37 °C. Doubling times were calculated from estimates of cell titre obtained from periodic A_{660} readings. The reported rates are the averages of experiments with two independent isolates of each strain (except for yU2-107 and yU2). With the exception of cultures at 37 °C, the growth rate in liquid was consistent with that observed on plates. It is possible that the temperature-sensitive growth defect is sensitive to differences in osmotic pressure.

experiment. Forty-five of the 120 nucleotides within the yU2 5' conserved domain are altered or missing in hU2 (ref. 11) and the putative 3' terminal stems differ in both size (11 against 17 base pairs) and sequence (90 of the terminal 97 nucleotides are different), indicating that the essential functions of the U2 snRNA are all accomplished by a comparatively small number of highly conserved residues. The surprising efficiency with which the human RNA performs in yeast now prompts us to ask whether other human RNAs can function in complementation experiments *in vivo*. The ability to assemble chimaeric spliceosomes would greatly facilitate efforts to understand the myriad interactions that occur within a functioning splicing complex. □

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A regular pattern of two types of 100-residue motif in the sequence of titin

S. Labeit*, D. P. Barlow†, M. Gautel‡, T. Gibson*, J. Holt§, C.-L. Hsieh||, U. Francke||, K. Leonard*, J. Wardale¶, A. Whiting¶ & J. Trinick¶#

*European Molecular Biology Laboratory, Meyerhofstrasse 1, 69 Heidelberg, FRG

†Research Institute of Molecular Pathology, Dr Bohr-Gasse 7, A-1030 Vienna, Austria

‡Max-Planck Institute for Medical Research, Jahnstrasse 29, 69 Heidelberg, FRG

§Macromolecular Analysis and Synthesis Laboratory, Temple University Health Sciences Center, Philadelphia, Pennsylvania 19140, USA

||Howard Hughes Medical Institute and Department of Genetics, Stanford University Medical Center, Stanford, California 94305-5428, USA

¶AFRC Institute of Food Research-Bristol Laboratory, Bristol BS18 7DY, UK

TITIN is the largest polypeptide yet described (relative molecular mass $\sim 3 \times 10^6$; refs 1, 2) and an abundant protein of striated muscle³. Its molecules are string-like⁴ and *in vivo* span from the M to Z-lines^{5,6}. I-band regions of titin are thought to make elastic connections between the thick filament and the Z-line⁵⁻⁷, thereby forming a third type of sarcomere filament^{8,9}. These would centre the A-band in the sarcomere and provide structural continuity in relaxed myofibrils¹⁰. The A-band region of titin seems to be bound to the thick filament, where it has been proposed to act as a 'molecular ruler' regulating filament length and assembly⁶. Here, we show that partial titin complementary DNAs encode a regular pattern of two types of 100-residue motif, each of which probably folds into a separate domain type. Such motifs are present in several evolutionarily divergent muscle proteins, all of which are likely to interact with myosin. One or both of the domain types is therefore likely to bind to myosin.

A λ gt11 cDNA expression library was constructed from rabbit psoas muscle and probed with antibodies against titin. The specificity of the antibodies was established by western blots

against whole muscle which showed binding only to titin (Fig. 1, and ref. 6). From a total of 7×10^5 cDNA clones screened with various mixtures of twelve monoclonal and three polyclonal antibodies, three immunoreactive clones were identified and confirmed in duplicate experiments¹¹. In further screening, each clone was shown to react with only one antibody. Clone T1 was identified by monoclonal antibody, CE12, which labels muscle at a position in the A-band $0.33 \mu\text{m}$ from the M-line⁶. Subsequently, T2, a larger cDNA encompassing T1, was isolated

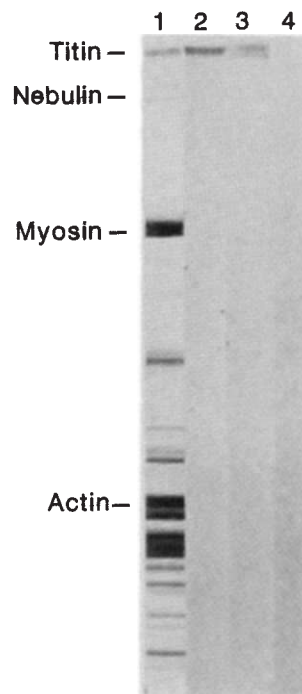


FIG. 1 Western blot showing that antibodies CE12 and MS2 bind only to titin when reacted against whole muscle. Splitting of the titin band is due to proteolysis⁴. Lane 1, Coomassie blue-stained gradient gel (3-10%); lane 2, CE12 blot; lane 3, MS2 blot; lane 4, pre-immune serum blot. Methods as described in ref. 6.

To whom correspondence should be addressed.

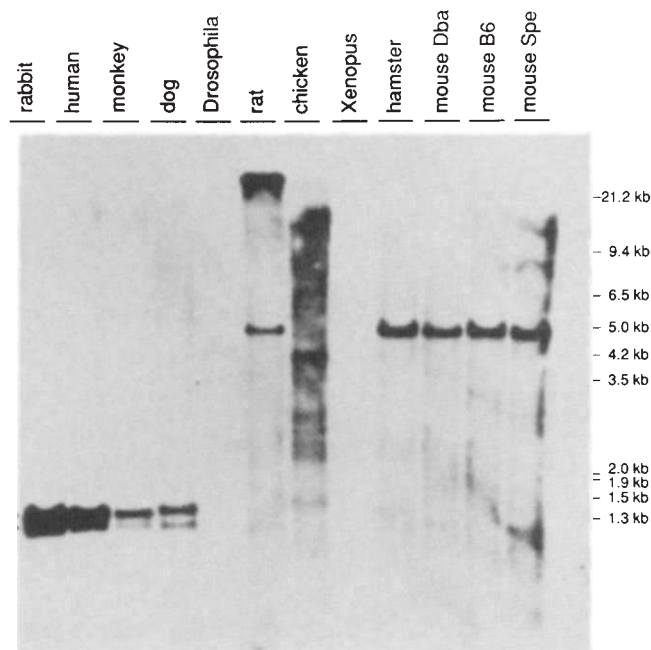


FIG. 2 Southern blot showing hybridization of T2 to genomic DNA from several species and several mouse strains (Dba, B6 and Spe). The strong signals and similar sizes of restriction fragment lengths suggest an unusual degree of conservation. Arrows mark bands of approximately 1.3 and 1.5 kb in rabbit DNA.

METHODS. Genomic DNA was prepared²⁴ from 10 different species, digested with various restriction enzymes, electrophoresed, transferred to nylon membrane and hybridized to a randomly labelled T2 probe²⁵ as previously described¹². Washings were performed at reduced stringency (40 mM NaP_i (pH 7.2), 1% SDS, 55 °C). The filter was exposed -80 °C overnight.

by screening a λ gt10 library with a ³²P-labelled T1 probe. Clones T3 and T4 were both identified by a polyclonal antibody, MS2, which was raised against a titin proteolytic fragment. Immunofluorescence indicated MS2 labels myofibrils at several positions in the sarcomere, and so the positions of sequences corresponding to T3 and T4 are not firmly established. That T1, T3 and T4 were not false positives from non-titin genes was confirmed by mapping them to the same section of the rabbit genome. Genomic DNA was digested with different restriction enzymes and separated by pulsed-field electrophoresis¹³. The smallest fragment to which all three cDNAs hybridized was 280 kilobases (kb), prepared using *Sal*I. As the cDNAs are probably from the same gene and were identified by two different titin-specific antibodies, the probability that the gene involved codes for a protein other than titin is remote. This gene is remarkably conserved; on Southern blots a T2 probe cross-hybridized to genomic DNA from all vertebrates tested; moreover, restriction fragments of similar size were observed (Fig. 2).

RNA species recognized by the cDNAs¹² were also consistent with titin. Titin has a characteristic distribution, being abundant in striated muscle but absent from smooth muscle or non-muscle tissues¹³. RNase protection¹⁴ assays with a T1 probe detected strong signals in psoas and heart muscle, but no signal in colon or ovary (smooth muscle) or in non-muscle tissues such as liver, kidney, brain or lung. On northern blots, T2 hybridized to a band that could not be resolved from the region of limiting mobility and had a lower mobility than either rabbit dystrophin messenger RNA (13 kb) or a 21-kb phage λ restriction fragment (Fig. 3). This is consistent with the ~100 kb mRNA required to encode a protein of M_r 3,000K.

Hybridization of a T2 probe to panels of DNA from Chinese hamster/human hybrid cell lines assigned the titin locus to human chromosome 2 q13-33. Interestingly, the locus of another myofibrillar protein, nebulin, is known to be in an overlapping region, q31-32, on this chromosome^{15,16}. Nebulin is also large (~700K) and may control thin-filament length¹⁷. The fact that the two genes are close together suggests their regulation may be coordinated, possibly to control the ratio of the proteins. In mouse, the titin gene was also mapped to chromosome 2, which contains the locus of a severe muscular dystrophy with myositis (*mdm*)¹⁸.

The sizes of T1, T2, T3 and T4 were 0.6, 1.8, 1.2 and 0.9 kb respectively, or ~4% of the coding sequence for the whole molecule. T2, T3 and T4 were then extended using the polymerase chain reaction (PCR) as described¹⁹, so that a total of 7 kb has now been accumulated in two roughly equal size sections. The first round of PCR amplification directed against the ends of T3 and T4 showed them to be separated by only 150 nucleotides, presumably due to antibody MS2 being polyclonal. Sequence analysis²⁰ of the aggregate sections, termed MS2 contig and CE12 contig, predicted a single long open reading frame in each case. The derived amino acid sequences consist of two classes of repeated motifs, termed I and II, each containing roughly 100 residues. Within each motif class,

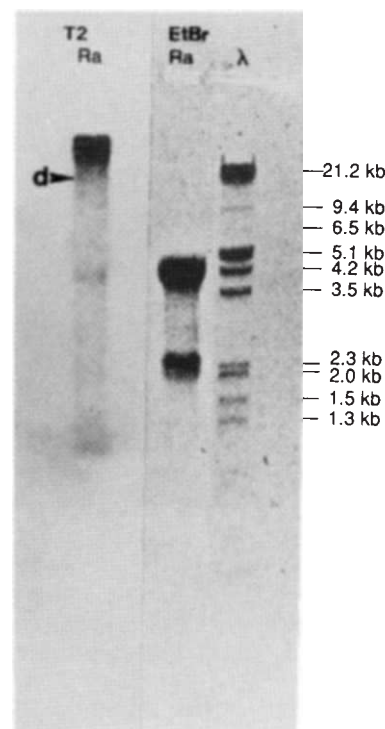


FIG. 3 Northern blot of titin mRNA. T2 hybridizes to an mRNA that cannot be separated from the region of limiting mobility (~25 kb). The arrow (d) marks the position recognized when the blot was rehybridized with human dystrophin cDNA probe 63.1. T2, radiolabelled T2 probe; EtBr, ethidium-bromide staining. Total rabbit RNA from leg; λ , *Eco*RI/*Hind*III digested lambda size markers, sizes indicated in kilobases (kb).

METHODS. Total cellular RNA was prepared from tissue using the guanidine isothiocyanate method²⁶ and 10 μ g samples were sized on formaldehyde gels²⁷. Outer lanes including an *Eco*RI/*Hind*III restricted lambda marker were removed and stained with EtBr. After transfer to nylon membranes the remaining lanes were hybridized to randomly labelled probes¹² of T2 or human dystrophin cDNA probe 63.1. Filters were exposed at -80 °C overnight.

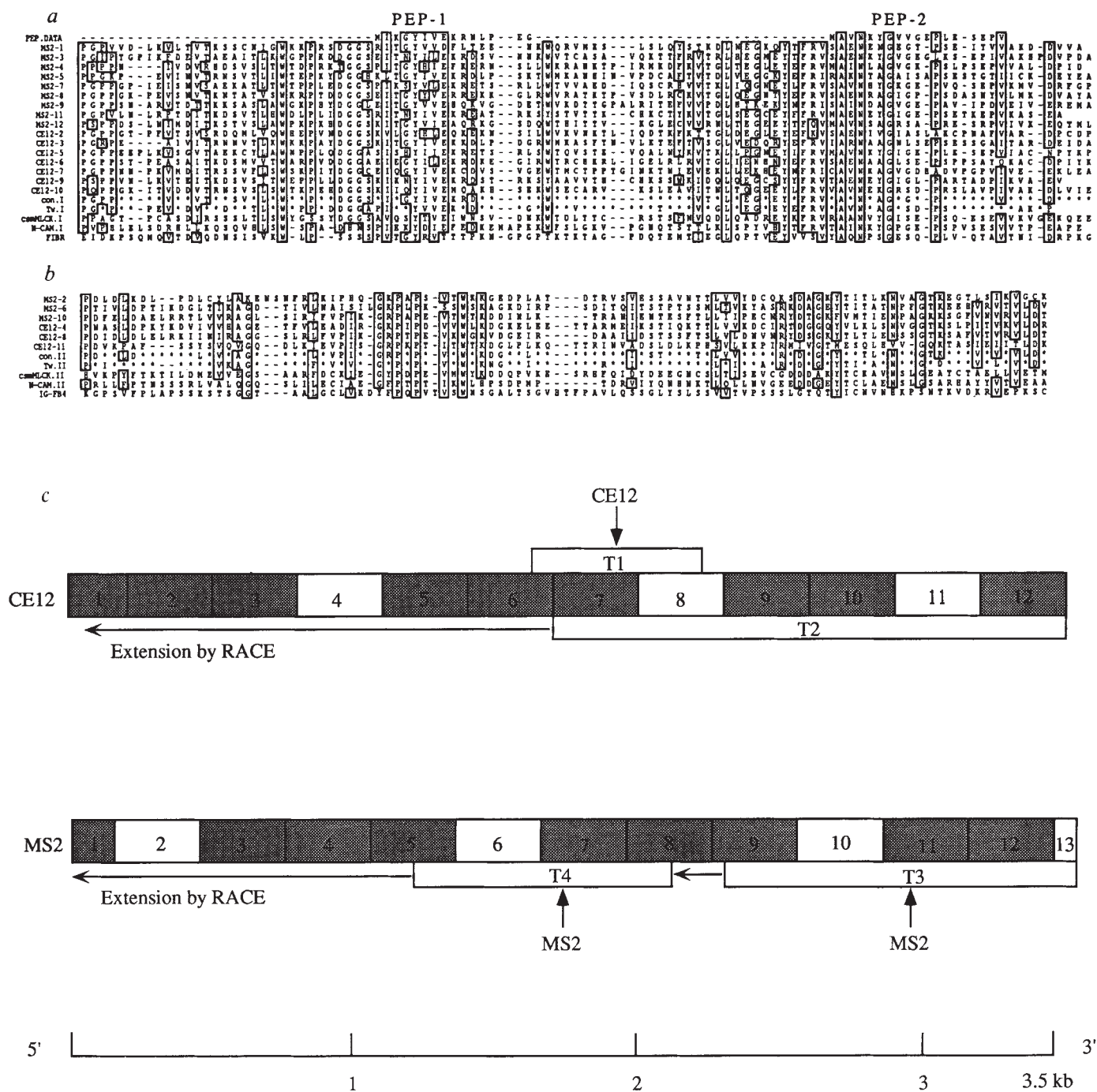


FIG. 4 Titin domain structure. *a*, Motif I alignments, using the single-letter amino acid code. Sixteen complete members of class I domains from the CE12 and MS2 cDNA contigs are shown. The consensus, con.I, is very similar to the consensus, tw.I, of class I motifs found in twitchin²¹. Similarities were also found with chicken smooth muscle myosin light chain kinase (csmMLCK), the extracellular section of N-CAM²⁸, and fibronectin²⁹. Domain numbers are from Fig. 4c. *b*, Motif II alignments. Six complete class II motifs are shown. Con.II, is similar to tw.II, the consensus of type II motif from twitchin²¹. Similarities were also found to the non-catalytic sections of csmMLCK^{22,23}, N-CAM²⁸, and the monoclonal antibody KOL heavy chain (for which the structure is determined to 1.9 Å³⁰). *c*, Class I (stippled boxes) and class II motif organisation in CE12 and MS2 cDNA contigs. (Assignment of MS2 domain 13 to class II is tentative, since only 9 residues have been determined.) The CE12 and MS2 contig nucleotide sequence data have been deposited in the EMBL database under accession numbers X17329 (CE12) and X17330 (MS2).

METHODS. Three walking cycles based on the anchored 'rapid amplification of a DNA ends' protocol¹⁹ were used to extend the T2, T3 and T4 cDNAs further 5'. Reverse transcribed mRNA from rabbit psoas muscle was tailed with terminal transferase and dGTP. Titin sequences were amplified by PCR for 40 cycles using synthetic oligonucleotides complementary to the region -20 to -40 bp downstream from the 5' end, and a dC15 mer for the 3' end. Amplified DNA sequences were subcloned into M13 and 8 recombinant clones each were sequenced. Approximately half of the clones had the correct -1 and -20 sequence and extended from the 5' end by ~500 nucleotides. To exclude PCR artefacts, at least two independent clones obtained by RACE were sequenced and shown to be identical. Sequences of the respective domains were aligned by inspection and allowing a maximum insertion of three residues. Residues conserved in >50% of all titin members are listed as the consensus sequences, con.I and con.II, and for twitchin tw.I and tw.II²⁰. Positions showing >60% conservation (scoring D or E, W or Y, L or F, and V or I as matches) are boxed (Prettyplot by P. Rice, EMBL). Peptides for sequencing were isolated from purified native titin⁴ (1 mg ml⁻¹ in 6M guanidine-HCl, 0.1N HCl) digested with cyanogen bromide (30 mg in 70 μ l acetonitrile) for 90 min at 37 °C and 18h at 4 °C. The digest was fractionated by reverse phase HPLC (silica C-18 column in 0.1% trifluoroacetic acid, eluted with acetonitrile) and selected peaks sequenced.

roughly half the residue positions are conserved (Fig. 4a, b). The protein sequences of two cyanogen bromide peptides from purified native titin⁴ clearly fit into the class I pattern (Fig. 4a). The arrangement of motifs in both contigs is -I-I-I-II-I-I-II-I-I-II- (Fig. 4c). As titin was previously suggested from electron microscopy to consist of a linear array of 43 Å globular domains each containing approximately 100 residues^{4,6}, each motif is likely to fold into a separate domain.

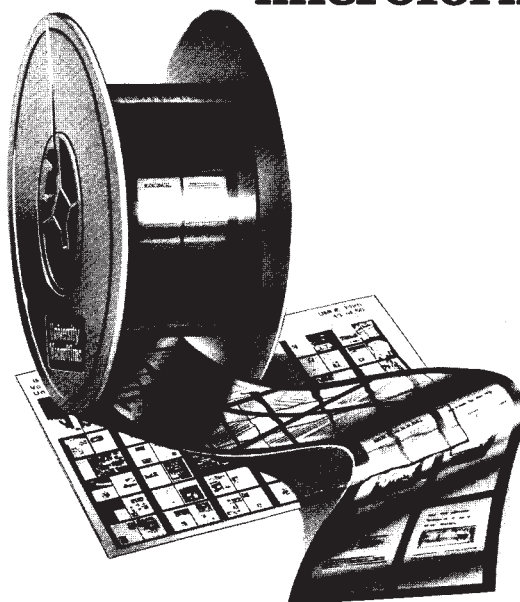
Class I and II are similar to sequences recently discovered in twitchin, a muscle protein from *Caenorhabditis elegans*. These motifs are respectively members of the fibronectin and immunoglobulin superfamilies²¹ which were thought to be exclusively extracellular, but now include a diverse range of intracellular muscle proteins. In addition to titin and twitchin, class I and II motifs are also found in chicken smooth muscle myosin light chain kinase (csmMLCK)²¹⁻²³, and in C-protein and 86K protein³², both from chicken³³. Of these muscle proteins, titin and twitchin are most similar; moreover, twitchin is also large (668 K) and has similar domain patterns to titin²¹. However, twitchin also contains sequence near its C terminus very similar to the catalytic domain of MLCK. It has therefore been suggested that twitchin controls contraction in *C. elegans*²¹. By contrast, assays and antibody experiments have not revealed evidence of a kinase domain in titin. It also seems unlikely that twitchin molecules span half the sarcomere in *C. elegans*, as they are substantially smaller than titin and sarcomeres here are longer than in vertebrates. These common features, despite the large evolutionary distance between *C. elegans* and rabbit, suggest a common ancestral gene, but the differences between the two molecules argue for different functions acquired during evolution. As each member of the intracellular group with class I and II motifs almost certainly interacts with myosin, it seems probable that either or both of these domain types can bind myosin. □

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